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13. ABSTRACT *(Maximum 200 Words)*

Quantitative tumor transduction represents a major limitation to the achievement of meaningful clinical results in cancer gene therapy protocols. Approaches directed towards the goal of enhancing or amplifying the effects of a genetic transduction event may further enhance the potential efficacy of cancer gene therapy strategies. One way to achieve this amplification effect would be via replication of the delivered viral vector. In this approach, a conditionally replicative competent virus would be utilized to selectively replicate within the transduced tumor cells and not in normal tissues. Adenoviral vectors possess the unique attribute with respect to the*in vivo* gene delivery recommending their employment as conditionally replicative vectors. It is our hypothesis that a conditionally adenovirus that would replicate selectively and specifically into tumor cells could be developed and utilized as an experimental tumor therapy modality for prostate cancer. In these initial studies, we have shown that improving the infectivity of adenoviral vectors dramatically augments the oncolytic potency of CRAD agents. The establishment of this key principal now feasibilize our original goal to improve the replicative specificity of the CRADs for carcinoma of the prostate.

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(4). INTRODUCTION

We have endeavored to improve the efficacy of conditionally replicative adenoviruses (CRAds) as therapeutic agents in the context of carcinoma of the prostate. Our initial goals related to developing the basis of tumor selective replication via exploiting the IL-6/IL6-R axis. In the course of our studies, however, we noted tumor cell resistance to adenovirus infectivity based upon deficiency of the primary Ad receptor, CAR. Such infectivity limits represented a major barrier to deriving the full benefit of CRAd agents, as effective amplification required target cell susceptibility to adenovirus infection. To address this issue, we altered adenoviral tropism via genetic capsid modification. In this regard, incorporation of an integrin targeting peptide within the HI loop of the fiber knob allowed CAR-independent gene transfer with the achievement of dramatic augmentations in adenovirus infectivity. On this basis, we modified an available CRAd, Ad5-Δ24, to incorporate such a targeting motif within the HI loop. In these studies, the infectivity-enhanced CRAd, Ad5-Δ24RGD, exhibited a dramatically augmented ability to achieve oncolysis of human prostate cancer cell lines compared to the unmodified CRAd Ad5-Δ24. Having addressed this major barrier to CRAd utility, it is currently rational to endeavor further steps to improve the replicative specificity of these agents for prostate cancer cells, as initially proposed. CRAd agents embodying these two capacities, infectivity enhancement and replicative specificity, will allow realization of the full therapeutic potential of replicative adenoviral agents.

(5). BODY

The focus of this proposal was the development of a novel therapeutic for carcinoma of the prostate employing the approach of oncolytic viruses. In this regard, adenoviruses can be engineered to replicate selectively within tumor cells whereby anti-tumor effects can be achieved directly via viral oncolysis. Such conditionally replicative adenoviruses (CRAds) have been employed in the context of a variety of neoplasms, including carcinoma of the prostate, and have been rapidly translated into the context of human clinical trials.

To be effective as anti-tumor agents, CRAds must accomplish specific objectives in the context of the target tumor. Specifically, the CRAd agent must achieve a high initial infectious innoculum within the target tumor cells, must replicate selectively within tumor cells, must lateralize effectively based on effective infection of bystander tumor cells, and finally, these steps must occur in the context of intact immune effector mechanisms.

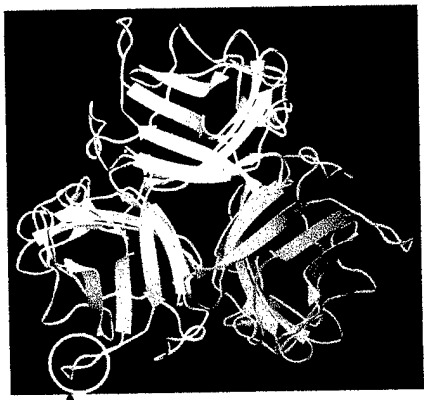
Based on these concepts, we initially endeavored an approach to develop the means for tumor selective replication. Our strategy was based upon the observation that IL-6 could transcomplement deleted adenoviral E1A and induce replicative oncolysis. **The transition of this strategy to the context of *in vitro* and *in vivo* models of carcinoma of the prostate was predicated upon the concept that the target prostate carcinoma cells were susceptible to adenoviral infection**. Such target cell susceptibility relates critically to the key CRAd requirements of high initial infectious innoculum and effective lateralization, noted above. Limitations in the realization of these objectives would clearly compromise the overall utility of CRAd agents for carcinoma of the prostate, irrespective of our success in achieving replicative specificity.

In this regard, studies in our laboratory had demonstrated a relative deficiency of the primary adenoviral receptor, coxsackie-adenovirus receptor (CAR), in the context of a number of tumor types. Furthermore, such CAR deficiencies rendered cellular targets relatively resistant to adenoviral vectors. Of note, these findings have been corroborated by a number of other groups. **On this basis, it was apparent that any attempts to employ adenoviral agents for cancer gene therapy would be confounded**. Such limits would be even more apparent in the context of CRAds, whereby target cell susceptibility to adenoviral infection is critical to the achievement of two key steps predicating oncolytic efficacy.

Based upon this recognition, we have endeavored steps to modify the tropism of adenovirus such that target cell infection could occur via non-native pathways. In this regard, we hypothesized that the ability to achieve "CAR-independent" infection would provide a means to circumvent functional deficiencies in the levels of the native adenovirus (Ad) receptor, thus allowing augmented infection of target tumor cells. We initially have achieved this end via retargeting complexes, which serve to route the adenovirus via heterologous receptors. Re-targeted adenoviral infection has been achieved via several target cell receptors including folate, EGF-R, CD40, EpCAM, and others. **These experiments have confirmed that the adenoviral infection may occur by virtue of non-CAR pathways. Most importantly, this method of retargeting adenoviruses has allowed enhancements of adenoviral infection of otherwise refractory target cells.**

The impracticalities of directly translating this method, based on a two component system, into human clinical context led us to explore alternate methods of altering adenoviral tropism as a means to achieve CAR-independent infection. In this regard, we endeavored to incorporated targeting ligands into the adenoviral capsid via genetic methods to realize an integrated system compatible with ultimate human use. Consideration of the structure of the adenoviral capsid served to identify a candidate locale for incorporation of heterologous targeting ligands within the HI loop of the fiber knob (Figure 1). Initial studies confirmed that short peptide motifs could be configured into this site without perturbation of the key structure/function parameters of the fiber. Most importantly, viable viruses could be rescued which contained heterologous peptide motifs within the HI loop, thus confirming this site as a propitious locale for ultimate incorporation of candidate targeting ligands.

A.



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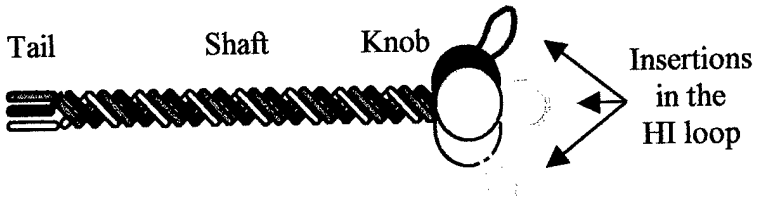

Figure 1: Genetic modification of the HI loop of the fiber knob domain. The knob trimer, viewed along the threefold symmetry axis (Reproduced from Xia, et al.). (B). Localization of targeting ligands within the fiber molecule.

We next sought to alter the tropism of adenovirus via this approach of genetic capsid modification employing the identified HI loop locale. To this end, the technique of phage library panning has provided a means of identifying cell specific ligands derived from both peptide and scFv phage libraries. For our initial experiments, we employed the peptide RGD4C which recognizes integrins of the $\alpha v\beta 3$ and $\alpha v\beta 5$ classes, frequently unregulated in epithelial tumors and tumor vasculature. Initial efforts were directed towards the derivation of a replication-incompetent adenoviral vector containing the RGD4C in the HI loop. This vector was then employed for gene transfer to tumor cell targets. Our studies demonstrated that the tropism-modified adenovirus was capable of achieving CAR-independent gene transfer as hypothesized. Of note, this CAR-independent viral routing allowed very dramatic enhancements of gene transfer to target cells (Figure 2). This was especially noteworthy in the context of primary tumor cells, whereby profound CAR deficiencies have been noted. These studies thus established that genetic capsid modifications, via the incorporation of targeting peptides within the HI loop of fiber, allowed a very effective means of circumventing target cell CAR deficiency.

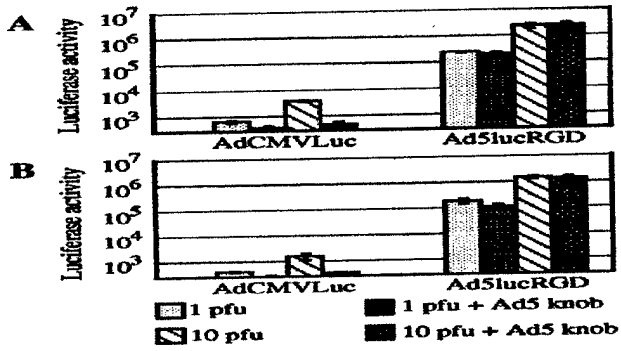

Figure 2: Infection of primary cells isolated from ascites obtained from ovarian cancer patients. Cells isolated from ascites of two (A and B) ovarian cancer patients were transduced with AdCMVLuc or Ad5lucRGD at MOI of 1 or 10 in the presence or absence of blocking Ad5 fiber knob protein. The data points represent the mean of three independent determinations.

In the aggregate, these studies suggested a facile method to enhance adenoviral infectivity for tumor target cells. As noted, effective infectivity is an essential attribute of CRAd agents, which predicates their utility as oncolytic agents. On this basis, we sought to determine whether infectivity enhancements of Ad, via tropism modifications, would improve the anti-tumor efficacy of CRAd agents. As an initial proof-of-principle, we employed an available CRAd agent, Ad5-Δ24. This adenovirus is deleted in the E1A gene such that it replicates conditionally in target cells with a dysfunctional RB/p16/E2F axis. For these studies, a variant of Ad5-Δ24 was derived which contained the RGD4C peptide within the HI loop

(Ad5-Δ24RGD). Target cells were then infected with Ad5-Δ24 and Ad5-Δ24RGD with direct analysis of oncolysis determined by crystal violet staining and XTT. **In these studies, it could be noted that the RGD modified version of the CRAd exhibited a significantly greater level of tumor oncolysis than the unmodified control adenovirus. These studies have thus established the key principle that adenoviral infectivity enhancement maneuvers can significantly improve the therapeutic utility of CRAd agents.**

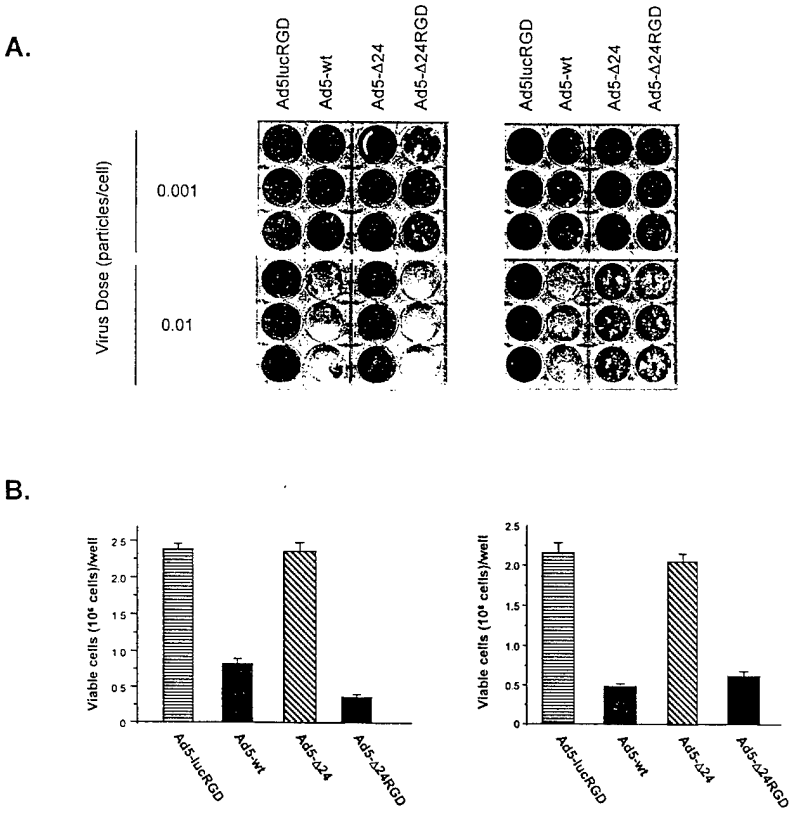

Figure 3: Oncolytic potency of the RGD-modified virus. (A). LNCaP cells were infected with low doses (0.01 and 0.001 viral particles/cell) of Ad5lucRGD, Ad5-Δ24, Ad5-Δ24RGD, and Ad5-wt. At 8 days post-infection, LNCap cells were fixed and stained with crystal violet. (B). Simultaneously cell viability was analyzed using a colorimetric assay based on XTT.

In summary, CRAd anti-tumor efficacy is linked to the ability to achieve tumor selective replication. Whereas this was the initial focus of our proposal, target cell resistance to adenovirus infection confounded our ability to directly endeavor this study. Of note, the ability of adenovirus to achieve an effective initial innoculum

of infection, as well as the ability to infect laterally, are also key predicates of CRAd utility. **Our studies herein have established the principle that infectivity enhancement of Ad can directly improve CRAd-mediated target tumor cell oncolysis. Having achieved this end, it is now rational to presently re-address the issue of tumor selective replication**. We hypothesize that a combination of the capacities of replicate specificity and CAR-independent infectivity will allow the derivation of CRAd agents for carcinoma of the prostate which embody an optimized therapeutic index.

(9). KEY RESEARCH ACCOMPLISHMENTS

- Demonstration of key biologic limits of current generation CRAd for carcinoma of the prostate relating to tumor cell CAR deficiency
- Generation of CRAd agents, Ad5-Δ24RGD, which cirrumvents these limits and posseses enhanced efficacy for carcinoma of the prostate

(10). REPORTABLE OUTCOMES

1. Reynolds PN and **Curiel DT**. Strategies to adapt adenoviral vectors for gene therapy applications:targeting and integration. *Cold Spring Harbor Laboratory Press*, 1999.
2. Dmitriev I, Kashentseva E, Rogers BE, Krasnykh V, and **Curiel DT**. Ectodomain of coxsackievirus and adenovirus receptor genetically fused to epidermal growth factor mediates adenovirus targeting to epidermal growth factor receptor positive cells. *Journal of Virology* 74:6875-6884, 2000.
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9. Casado SE, Nettelbeck DM, Gomez-Navarro J, Gonzalez Baron M, Siegal GP, Barnes MN, Alvarez RD, and **Curiel DT**. Transcriptional targeting for ovarian cancer gene therapy. Submitted, *Gynecologic Oncology*, 2000.
10. Wesseling JG, Yamamoto M, Adachi Y, Bosma PJ, van Wijland M, Blackwell JL, Li H, Reynolds PN, Dmitriev I, Vickers SM, Huibregtse K, and **Curiel DT**. Midkine and cyclooxygenase-2 promotors are promising for adenoviral vector gene delivery of pancreatic carcinoma. Submitted, *Clinical Cancer Research*, 2000.
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(10) CONCLUSION

This study established an approach to improve the efficacy of conditionally replicative adenoviruses (CRADs) for prostate cancer gene therapy. These findings should have direct bearing upon CRAd trials for this disease whereby this new class of advanced generation adenoviral agents may improve therapeutic ends achieved via CRAd agents.

(11) APPENDICES

6

Strategies to Adapt Adenoviral Vectors for Gene Therapy Applications: Targeting and Integration

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For the effective application of gene therapy strategies to human disease, Anderson suggested that certain criteria should be met; namely, that vectors should deliver a therapeutic gene specifically to a target cell, that resultant gene expression should be at an appropriate level and for an appropriate period of time, and that delivery and expression of the therapeutic gene should be achieved within an acceptable safety margin (Anderson and Fletcher 1980). These criteria remain largely unmet. However, in recent years, disappointment in the results of clinical trials has forced a refocusing on the basics of vector design, resulting in steady advancements in vector technology that now show promise for more successful gene therapy.

Development of vectors that have in vivo efficacy is critical because many diseases for which gene therapy can be rationally considered require direct in situ gene delivery and cannot feasibly be addressed by an ex vivo approach. Replication-incompetent adenovirus is a potential candidate vector for clinical gene therapy based on several key attributes that include ease of production to high titer, infection of both dividing and nondividing cells, and systemic stability, which has allowed for efficient in vivo gene expression (Brody and Crystal 1994). However, the virus has several important limitations including its widespread tropism, stimulation of inflammatory and immune responses, and short-term transgene expression (Yang et al. 1995, 1996; Tomko et al. 1997). This chapter focuses chiefly on the issue of targeted gene delivery to address the limitations brought about by native viral tropism. To date, several groups have sought to exploit the fundamental advantages of adenovirus

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by using it in specific contexts where the recognized limitations were felt to be less important. For example, it was thought that the issue of the widespread tropism of the virus could be circumvented by administering the vector by direct injection, particularly in the context of tumors. However, in phase I human trials, dissemination beyond the injected site has been found (D.R. Wilson, unpubl.). Application to "compartmentalized" disease has also met with problems. For example, poor transduction efficiency has been noted following administration into the pleural space for therapy of mesothelioma (Esandi et al. 1997; S.M. Albelda, unpubl.), and in the peritoneum, effective use of antitumor gene therapy has been limited by concurrent transduction of the liver with subsequent toxicity (Yee et al. 1996). Further limitations have arisen in the application to pulmonary disease. Here, prior clinical experience had indicated that the virus had a natural tropism for the respiratory tract, thus direct administration of vector to the airways for cystic fibrosis therapy seemed a rational approach Crystal et al. 1994; Zabner et al. 1993, 1996; Knowles et al. 1995; Bellon et al. 1997). In reality, the levels of transduction achieved were lower than expected because differentiated airway epithelial cells lack sufficient adenoviral receptors and the integrins required for viral internalization (Grubb et al. 1994; Goldman and Wilson 1995; Goldman et al. 1996; Zabner et al. 1997). Thus, even in these apparently favorable anatomical locations, there is a strong case for developing a vector with cell-specific targeting properties. Despite the limitations of adenovirus, its basic advantages, in particular its in vivo efficacy, justify using this virus as a starting point in the development of improved vector systems.

ADENOVIRAL ENTRY PATHWAY

Strategies for the retargeting of viral vectors were first applied to retroviruses and were based on a sound understanding of viral entry mechanisms (Cosset and Russell 1996). The entry mechanisms of adenovirus, including the recent identification of primary adenoviral receptors, are now well understood and allow for a rational approach to the targeting of adenoviral vectors (Fig. 1).

The adenovirus is an unenveloped icosahedral particle with 12 fibers projecting from the surface (Shenk 1996). During the assembly phase of viral replication, fiber monomers trimerize in the cytoplasm, then bind to a viral penton base protein that is subsequently incorporated into the viral capsid. At the distal tip of each fiber monomer is a globular region referred to as the knob domain. It is this knob region which binds to cellular adenoviral receptors, initially anchoring the virus to the cells. Two

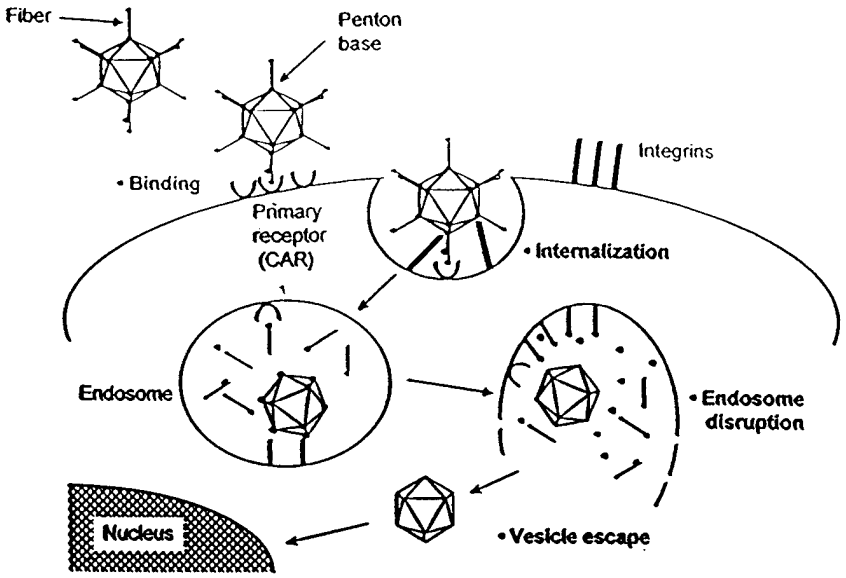

Figure 1 Adenovirus infection: Binding and entry pathway.

cellular receptors for adenovirus have recently been described. The coxsackie/adenoviral receptor (CAR) (Bergelson et al. 1997; Tomko et al. 1997) binds both adenovirus and group-B coxsackie viruses. The murine and human receptors are made up of 365 and 352 amino acids, respectively, and are 91% identical. The extracellular region appears to contain two immunoglobulin-like domains. In a separate report, viral binding to the α2 domain of major histocompatability complex class I has also been shown (Hong et al. 1997). Following attachment, viral entry requires a second step, which involves the interaction between Arg-Gly-Asp (RGD) motifs in the penton base with cell surface integrins αvβ3 or αvβ5, which then leads to receptor-mediated endocytosis of the virion (Wickham et al. 1993). In the endosome, the virus undergoes a stepwise disassembly and endosomal lysis occurs (a process mediated by the penton base and low endosomal pH), followed by transport of the viral DNA to the cell nucleus. This endosomolysis step is critical for efficient gene delivery, and the ability of the adenovirus to effect endosomal escape is one of the key factors in its efficiency as a vector. Importantly, viral entry and endosomal escape are functionally uncoupled (Michael et al. 1993); thus, entry via a nonnative, cell-specific pathway does not appear to compromise downstream delivery of DNA to the nucleus. Based on the foregoing, a logical place to start in the development of a targeted adenoviral vector is manipulation of the knob domain.

Several groups are now developing strategies to impart targeting ability to adenoviral vectors. The strategies currently being used fall under two broad headings: immunological and genetic.

IMMUNOLOGICAL RETARGETING

Immunological retargeting strategies are based on the use of bispecific conjugates, typically a conjugate between an antibody directed against a component of the virus and a targeting antibody or ligand. True targeting requires a simultaneous abolition of native targeting and introduction of new tropism; thus, Douglas et al. (1996) developed a neutralizing monoclonal antibody against the knob region of adenovirus. This was achieved by immunizing mice with adenovirus and recombinant knob protein, then developing hybridomas that produced antibodies capable of neutralizing native adenoviral infection (as determined by a cytopathic effect assay using HeLa cells). The Fab fragment of an antibody generated in this way (1D6.14) was then conjugated to folate to effect targeting to the folate receptor. Folate was chosen because the folate receptor is up-regulated on several tumor types and the receptor internalizes after ligand binding (Weitman et al. 1992). Using this conjugate, adenoviral infection was redirected away from the native receptor to the folate receptor (Fig. 2). The gene transfer efficiency of this approach was approximately 70% of that seen with the native virus, which contrasted to the experience with retroviral retargeting where redirection had generally resulted in a dramatic fall in infectivity (Cosset and Russell 1996). Although the binding of the complex to cells was clearly mediated by folate, the mechanism of viral internalization was not established. In this regard, folate is normally internalized by potocytosis, and enters the cell via a caveolus (Anderson et al. 1992). Normally, the size of this caveolus would be too small to encompass the folate-virus complex, but whether it could enlarge under these circumstances or whether viral entry was effected by the usual integrin-mediated pathway is unknown. Using a slightly different approach, Wickham et al. (1996) developed an adenovirus that contained a FLAG epitope, DYKDDDDK, introduced into the penton base region, and then used a bispecific antibody directed against FLAG and αv integrins to direct binding to integrins on endothelial and smooth muscle cells. Because the retargeting bispecific conjugate was larger than the viral fiber, it was hypothesized that the conjugate would be functionally available for binding by extending outward past the knob domain. In this way, by using integrins as the attachment target on these cells that express low levels of CAR, gene delivery was enhanced.

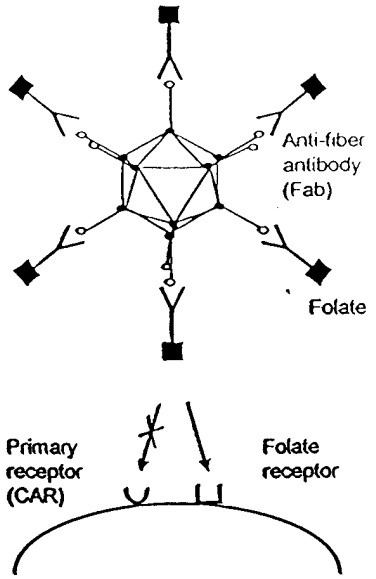

Figure 2 Schema for altering adenoviral tropism by an immunological targeting approach. A retargeting moiety formed by conjugating folate to the Fab fragment of an antiknob antibody is used to direct adenoviral binding to the folate receptor.

A further development in the immunological retargeting approach has been reported by Watkins et al. (1997). This group generated a bacteriophage library displaying single chain antibodies (scFvs) derived from the spleen of a mouse immunized against adenoviral knob. From this library a suitable neutralizing antiknob scFv was isolated, then a fusion protein between this and epidermal growth factor (EGF) was produced. This "adenobody" was then successfully used to retarget adenoviral gene delivery, resulting in enhanced transduction of EGF receptor (EGFR) expressing cells. Interestingly, this study showed that retargeting in this way appeared to bypass the need for the penton base–cell integrin interaction for internalization of the virus. This was shown by using an excess of an RGD-containing peptide that competes with penton for binding to integrins. This peptide reduced native viral transduction but had no effect on the transduction levels achieved with the retargeted vector complex, thus implying that in the latter case, viral entry was achieved by EGF receptor-mediated internalization. Taking a different approach to EGF receptor targeting, we have used a conjugate between 1D6.14 Fab and a monoclonal antibody that binds to EGFR (mab 425) (Murthy et al. 1987; Wersall et al. 1997). Using this approach, we have demonstrated retar-

geted delivery to two murine fibroblast cell lines, one stably expressing human EGFR, the other expressing a mutant of EGFR that does not internalize (C.R. Miller., unpubl.). Therefore, depending on the target selected, one may be able to exploit internalization mechanisms of either the targeted receptor itself or, if a noninternalizing target is selected, to use the native integrin pathway. The potential for overcoming a lack of integrins in some settings, or the ability to target to noninternalizing cell surface molecules if integrins are sufficient, implies a very broad potential applicability of retargeted vectors.

Following the initial demonstration that immunological retargeting of adenovirus could be achieved, further studies have explored the potential for therapeutic application of this approach. These applications might be considered in the context of several worthwhile goals of targeting with differing levels of stringency, including general nonspecific enhancement of delivery to a broad range of cell types, transduction of previously untransducible cells (relevant to both ex vivo and in vivo applications), targeting to enhance gene delivery and potentially reduce toxicity in a loco-regional or compartmental context, and cell-specific gene delivery following intravenous administration of vector.

At the lowest level of stringency, retargeting approaches to achieve enhanced gene delivery, even if in the absence of a clear specificity advantage, are worthwhile in compartmentalized disease contexts. Such approaches at the very least should allow for the use of lower doses of virus, thus potentially reducing direct viral toxic effects, dissemination from the administration site, and innate immune responses, which are clearly dose-dependent. In this regard, we have used basic fibroblast growth factor (FGF2) as a targeting ligand, based on the knowledge that FGF receptors are upregulated in a number of tumor types. A conjugate between 1D6.14 Fab and FGF2 was used to retarget adenoviral infection of a number of different tumor lines with varying baseline levels of susceptibility to adenoviral infection. Enhancements in transduction from two- to greater than tenfold were seen (Rogers et al. 1998).

With regard to the transduction of resistant cells, Kaposi's sarcoma (KS) is an example of a disease where gene therapy applications have been limited in part because of the poor transducibility of this tumor (J.A. Campain, unpubl.). Goldman et al. (1997) used the FGF2 retargeting approach to investigate retargeted gene delivery to previously untransfectable KS cell lines. The results demonstrated a dramatic increase in transduction. Furthermore, the potential therapeutic utility was shown by transfecting cells with a retargeted adenovirus carrying the gene for herpes simplex thymidine kinase (AdCMVHSV-TK). These cells were then

far more susceptible to killing by the subsequent administration of the prodrug ganciclovir than cells that had been infected with the untargeted virus. T lymphocytes are another cell type that is resistant to adenoviral infection, due to a lack of both CAR and αv integrins. Thus, Wickham et al. (1997a) successfully retargeted adenoviral vectors by using a conjugate between the anti-FLAG antibody and anti-CD3, thereby achieving significantly enhanced transduction. Thus, this study indicates the benefits of targeting can also be exploited in contexts relevant to ex vivo therapy.

In view of the direct cytotoxic effects of adenovirus, the ability to increase the number of transduced cells without resorting to an increase in viral dose is extremely important. For example, in the vasculature, where transduction is limited by a relatively low level of CAR expression (Wickham et al. 1996), transduction efficiency increases with escalating viral dose over a fairly narrow range, then dramatically falls as cytotoxicity supervenes and leads to a loss of infected cells. Schulick et al. (1995a) found a maximal transduction efficiency in vascular smooth-muscle cells of approximately 40% with 5×10^{10} pfu, which decreased to 0 at 10^{11} pfu. Similar results were also found in the endothelium (Schulick et al. 1995b). Using the bispecific anti–FLAG-anti-integrin approach, Wickham et al. (1996) achieved a seven- to ninefold enhancement of endothelial cell transduction. As FGF receptor expression is up-regulated in proliferating vasculature, and is thus relevant to a number of pathological processes including tumor angiogenesis, we examined the effect of FGF2 targeting of adenoviral gene delivery to proliferating endothelial cells. Here, an approximate 30-fold enhancement in luciferase expression was seen. Flow cytometry analysis of cells transfected with a β-galactosidase-encoding vector demonstrated that FGF2 retargeting led to both an increase in the number of transduced cells and an increase in the amount of gene expression per cell. In contrast, when FGF2 retargeting was used in the infection of quiescent, confluent cells, transgene expression was actually reduced, thus indicating a degree of cell-specific targeting based on the level of expression of the targeted receptor (P.N. Reynolds, unpubl.).

Toxicity at high viral doses has also been seen in murine carcinoma models, where escalating the dose of a herpes simplex thymidine kinase (HSV-TK)-encoding virus eventually led to deaths from toxicity before complete tumor eradication could be achieved (Yee et al. 1996). Also, in the context of HSV-TK, increasing the amount of transgene expression per cell (rather than the number of transduced cells) by manipulation of promoters did not increase therapeutic effect once a threshold level of

expression was achieved (Elshami et al. 1997). Thus, Rancourt and colleagues investigated the use of FGF2 retargeting of AdCMVHSV-TK in a murine model of ovarian carcinoma (C. Rancourt, unpubl.). First, FGF receptor expression on the ovarian carcinoma line SKOV3.ip1 was confirmed by radiolabeled FGF binding. Then, enhancement of adenovirally mediated gene delivery to these cells using FGF2 retargeting in vitro using the luciferase reporter gene was demonstrated. Next, validation of the retargeting approach in vivo was obtained. Tumors were established in nude mice by intraperitoneal inoculation with SKOV3.ip1 cells, followed 5 days later with a peritoneal injection of either AdCMVLuc alone or with FGF2 retargeting. Mice were sacrificed and luciferase expression in the tumors was quantified. Tumors from the mice that had received the retargeted vector had tenfold greater level of luciferase expression. Thus, these results established that the immunological retargeting was efficacious in vivo. Tumors were then established in mice as before, followed by intraperitoneal injection of either placebo, AdCMVHSV-TK alone, or AdCMVHSV-TK with FGF2 retargeting (with viral doses of 10^8 and 10^9 pfu). Mice were then divided into two groups and received either ganciclovir or placebo for 14 days and were monitored for survival. The mice that did not receive ganciclovir had no survival advantage over the mice that received no gene therapy. When the survival curves for the mice who received ganciclovir were analyzed, two important results emerged (Fig. 3). First, there was a statistically significant increase in survival with FGF2 retargeting compared to the untargeted vector at each dose of virus. Second, the survival curve for the mice treated with 10^8 pfu of virus with FGF2 retargeting was the same as that for 10^9 pfu of untargeted vector. Thus, FGF2 retargeting enabled a tenfold reduction in viral dose for the same therapeutic outcome. These results indicate a potential for increasing the clinical utility and therapeutic index of adenoviral vectors by using a retargeting approach.

Successful targeting following intravenous administration of a targeted adenoviral vector has yet to be reported. In this context, one of the major hurdles to overcome is hepatic uptake of injected virus, which accounts for greater than 90% of the injected dose. While it was initially considered that this problem was predominantly due to nonspecific uptake related to hepatic reticulo-endothelial functions, more recent evidence suggests that there may be a receptor-mediated component and that this may be potentially addressed by modification of native viral tropism. To this end, Zinn et al. (1998) investigated the in vivo distribution of technetium-labeled adenovirus serotype 5 (Ad5) knob. They found that the majority of it localized to the liver within 10 minutes of injection. This

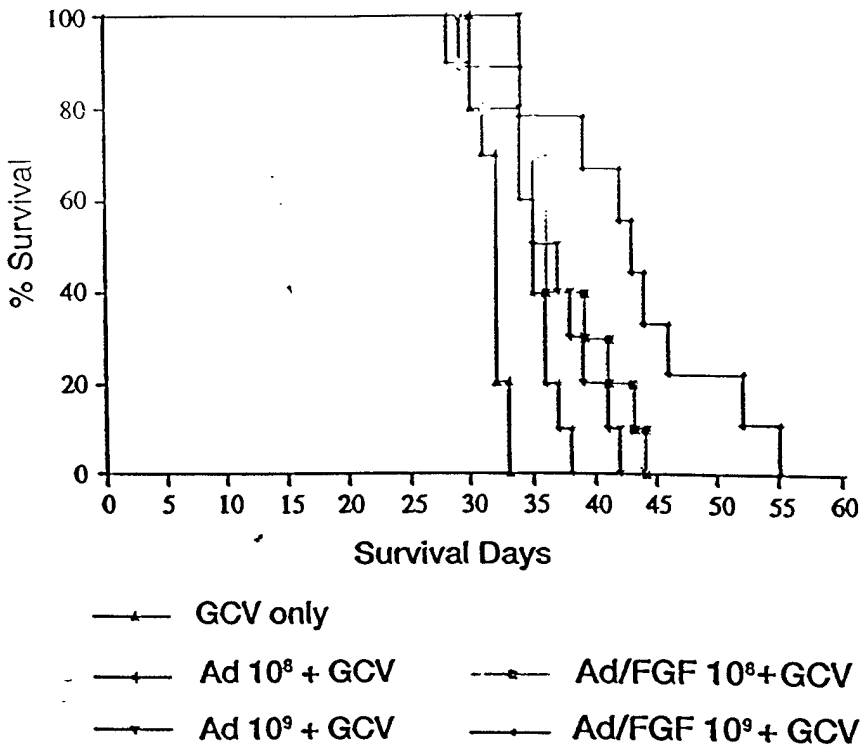

Figure 3 Retargeting adenovirus to enhance therapeutic gene delivery improves survival in a murine model of ovarian carcinoma. Mice with peritoneal ovarian tumors received intraperitoneal injections of placebo or adenovirus carrying the herpes simplex thymidine kinase gene (10^8 or 10^9 pfu)–either unmodified virus (Ad) or virus complexed to a retargeting moiety (formed by conjugating FGF2 to antiknob Fab) (Ad/FGF). Mice were then treated with ganciclovir (GCV) and survival monitored. Mice treated with retargeted adenovirus had a significant increase in median survival. Ad(10^8 pfu) vs. Ad/FGF(10^8 pfu), $p = 0.0025$; Ad(10^9 pfu) vs. Ad/FGF(10^9 pfu), $p = 0.007$.

localization could be inhibited by prior injection of an excess of unlabeled Ad5 knob, but not by an excess of serotype 3 (Ad3) knob (which binds to a different receptor), thus indicating the specificity of the hepatic uptake. When labeled Ad5 knob was complexed with 1D6.14 Fab prior to injection, hepatic uptake was markedly reduced, providing further evidence of the receptor-dependent nature of the uptake and indicating a degree of stability of the Fab to knob bond in the bloodstream.

The developments using immunological retargeting strategies have established a number of important principles. Modification of tropism

has successfully been achieved, indicating that true cell-specific delivery is possible. The evidence to date suggests that limitations in transduction due to either a deficiency in CAR, αv integrins, or both, may be overcome by retargeting. Efficiency of gene delivery with retargeted complexes is not only comparable to wild-type vector, but in many cases has resulted in a substantial improvement in gene delivery, which is itself a worthwhile goal. Retargeted complexes are efficacious in vivo, at least in a compartmental context. The use of a retargeted vector has been shown to enhance a therapeutic endpoint and finally, the limitations of intravenous application of these vectors, imposed by hepatic uptake of virus, appears, at least in part, to be a receptor-mediated phenomenon and may therefore also be overcome by retargeting. The full potential of immunological retargeting, however, is yet to be defined and there are certain practical and theoretical limitations to this approach. Large-scale production of bispecific antibody conjugates of consistent configuration is difficult when using the hetero-bifunctional cross-linkers that have so far been reported. Also, clearance of retargeting complexes and activation of the complement system may limit in vivo application. Although further protein engineering refinements such as the fusion protein "adenobody" approach may address some of these issues, the stability of the targeting complex-virion bond following systemic delivery remains a concern, especially when attempting intravenous administration. Thus, development of another approach, genetic retargeting, is also being pursued.

GENETIC RETARGETING

In view of the practical and theoretical limitations of the immunological approach to retargeting mentioned previously, development of targeted vectors by genetic manipulation of the virus itself has proceeded alongside the immunological strategies. In addition, immunological targeting approach is not likely to be sufficient for application to the controlled replicating viral vector systems being developed to improve gene delivery to malignant tumors. In these systems, the need for precise targeting of both the initial viral dose and the progeny viruses will be particularly important and only achievable by genetic modifications.

Based on the knowledge of native viral binding, a rational place to begin in the development of genetically targeted vectors is with the knob domain, and most strategies have so far focussed on this region. The question of whether viral tropism could be modified genetically was initially addressed by Krasnykh et al. (1996). A chimeric adenovirus con-

taining the serotype 3 knob on the Ad5 fiber shaft and capsid was produced by homologous recombination in 293 cells, using a modification of the shuttle and rescue plasmid technique developed by Frank Graham (Graham and Prevec 1991). Because Ad5 and Ad3 recognize different receptors, the tropism of the chimeric vector could be assessed by blocking infection of cells with an excess of free Ad5 or Ad3 recombinant knob. This confirmed that an "Ad5" vector possessing Ad3 tropism had been produced. A similar strategy was reported by Stevenson et al. (1997), who also demonstrated differences in transduction efficiency of various cell lines depending on whether wild-type or chimeric fiber vectors were used. Following the initial proof of principle, the incorporation of specific targeting ligands has been investigated.

When modifying the knob domain, there are important structural constraints that must be addressed. Because it is essential for fibers to trimerize to allow attachment to the penton base for subsequent capsid formation, any modification to the fiber must not perturb trimerization (Novelli and Boulanger 1991). Incorporation of a small ligand (gastrin releasing peptide) at the carboxy-terminal of fiber, and subsequent generation of trimers of this chimeric fiber, was initially reported by Michael et al. (1995). It has since been discovered that there are limits to the size of peptides that can be used in this way. Although the limits probably relate to the actual sequence used rather than to its length alone, it appears that trimerization is much less likely to occur with ligands longer than 25–30 amino acids. On the other hand, deletion of eight amino acids from the carboxyl terminus leads to failure of trimerization. Despite these limitations, the addition of small peptide ligands may have utility. For example, we have recently added a moiety containing six histidine residues (6-His) to the knob carboxy-terminal, successfully rescued the virus, and confirmed the binding availability of the 6-His moiety by using nickel column chromatography. This virus may be used to address the concerns regarding the stability of the bond between adenoviral vectors and immunological retargeting complexes. Theoretically, using an immunological retargeting complex containing an anti-6-His antibody, followed by the use of the nickel monoperoxyphthalic acid (MMPP) technology, might allow the formation of a stable covalent bond between the virus and the targeting complex (Fancy et al. 1996). Wickham et al. (1997b) recently reported a number of carboxy-terminal modifications, including one containing an RGD motif (21 amino acids) for the purpose of targeting to cell-surface integrins, and one containing seven lysine residues for the purpose of targeting to cell surface heparan sulfates. Production of a virus with longer carboxy-terminal additions was also attempted. A virus con-

taining a 32-amino-acid peptide for targeting the laminin receptor was produced, but it failed to bind its target receptor, whereas a virus containing a 27-amino-acid E-selectin-binding peptide could not be produced. Neither of the successfully produced viruses had any modification to native tropism; thus, their potential application is in the context of enhancing delivery to otherwise poorly transfectable cells, rather than true cell-specific targeting. Nevertheless, enhanced vascular delivery using the polylysine virus was demonstrated in vivo in a vascular injury model and may have utility in the therapy of angioplasty restenosis. The RGD containing virus was shown to enhance gene delivery to endothelial and smooth muscle cells in vitro.

In view of the limitations involved in attaching ligands to the carboxyl terminus, other regions of the knob may ultimately prove to be better sites for ligand incorporation. In this regard, we have investigated the HI loop region of the knob. X-ray crystallographic modeling of the three-dimensional structure of trimeric knob indicates that the HI loop is located on the outer aspect in an area potentially available for interaction with receptors (Fig. 4) (Xia et al. 1994, 1995). Also, this region does not appear to be directly involved in trimerization, contains mostly hydrophilic amino acids, and is different lengths in different Ad serotypes, suggesting that there may be less rigid structural constraints than at the carboxyl terminus. As initial proof of concept, we have successfully incorporated a FLAG epitope into the HI loop (Krasnykh et al. 1998), using a technique of recombinant virus generation involving homologous recombination in *Escherichia coli* (Chartier et al. 1996). Affinity binding to an M2 matrix column confirmed that this epitope was available for binding in the context of the intact virion. Other ligands with more relevant targeting potential have now been incorporated, including a cyclic RGD peptide (which has affinity for tumor vasculature) (Pasqualini et al. 1997) and somatostatin. The size constraints of ligand incorporation at this site are yet to be determined, and thus the incorporation of large ligands such as EGF and scFvs is currently being investigated. However, it is likely that the sheer size of an scFv will require an alternate strategy such as complete replacement of the entire knob domain.

Ultimately, for true targeting to be achieved, modification to ablate native tropism will need to be addressed. It may be that incorporation of large ligands into the HI loop will simultaneously ablate native tropism by steric hindrance; however, if this is not the case then further modifications will be required. In this regard, receptor-binding epitopes within the knob that may be suitable for mutagenesis strategies have been identified (Hong and Boulanger 1995). Clearly, if complete replacement of

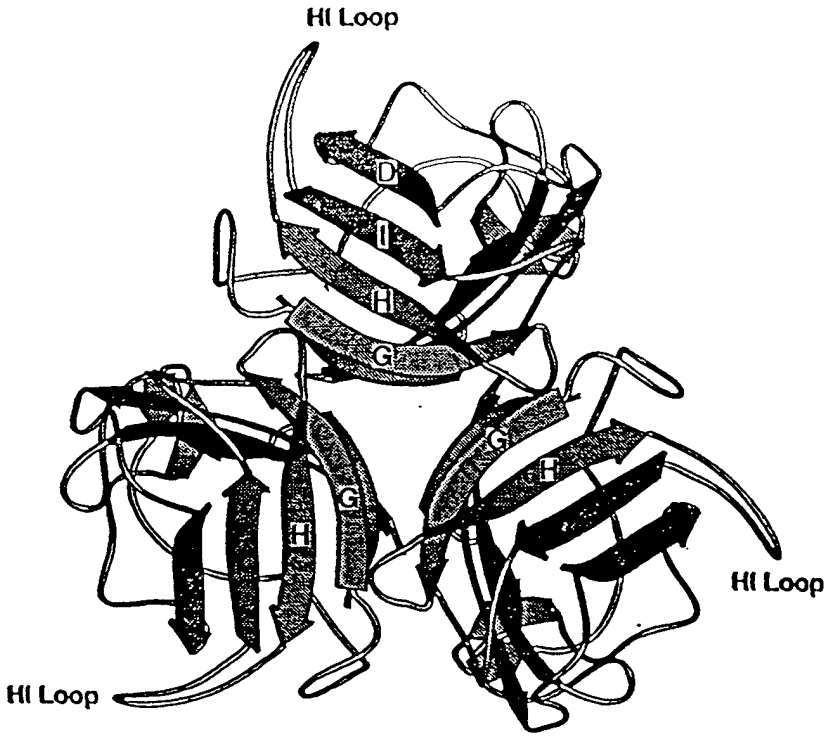

Figure 4 The knob trimer, showing the position of the HI loop. (Reprinted, with permission, from Xia et al. 1995 [copyright Springer-Verlag].)

the knob with a targeting and trimerization moiety could be achieved, this would simultaneously ablate native tropism. Of course, an integral part of any such strategy will be the use of permissive cell lines possessing the relevant target receptor to allow rescue and propagation of the virus.

Genetic modification strategies are not limited to the fiber. Wickham et al. (1995) introduced modifications into the penton base for targeting to cell-specific integrins. Hexon capsid proteins might also be exploited for targeting. An attractive aspect of this approach is the number of hexon proteins, 720, compared to the 36 knob regions; thus, hexon modification might have the potential for higher affinity binding. Such a strategy will still need to take into account the need to ablate native knob-dependent binding and address the stoichiometric issues relating to the fiber projecting out from the capsid, to ensure physical accessibility of the ligands introduced into the hexon.

As progress is being made in the development of retargeted vectors,

the importance of identifying truly cell-specific ligands has been highlighted. Although there are many established ligands and antibodies that may be candidates in certain settings, in many cases, such as mature airway epithelium, truly specific targets have yet to be discovered, thus requiring further target definition. This is especially relevant in the context of those genetic retargeting strategies that attempt to target with small peptide ligands. In this regard, the use of bacteriophage panning techniques have shown potential utility for target definition (Barry et al. 1996; Pasqualini and Ruòslahti 1996; Pasqualini et al. 1997). Bacteriophage can be engineered to express peptide sequences of various lengths and configurations (e.g., linear or cyclic) on their surface. Libraries of phage can be generated that express all possible sequences of peptide of a defined length or configuration. Using this library, one can pan against target proteins, cells, or even organs and tissues in vivo. By isolating the phage from the library that have affinity for the target of interest, serial rounds of panning can ultimately identify peptide sequences that show particular affinity for the target. For example, using this approach in vivo, Pasqualini and colleagues (1997) have identified a double-cyclic RGD-containing peptide with particular affinity for tumor vasculature. Similar strategies are also being used to define scFvs with targeting potential (Neri et al. 1997).

It has clearly been shown that viral tropism can be modified by genetic strategies. However, concurrent ablation of native tropism has not yet been achieved. The targeting ligands that have been successfully introduced at this stage are limited to short peptides, and incorporation of larger ligands may require new approaches. Nevertheless, progress in this area has been rapid and the results to date, coupled with the immunological retargeting results, indicate that development of a systemically stable, cell-specific vector is a very realistic aim.

INTEGRATION

The use of adenovirus as a vector has other important limitations apart from issues of targeting. One of the problems is the transient nature of the gene expression achievable with these vectors. While this may not be a central issue in the context of cancer gene therapy, where transient expression of a toxin gene may in fact be advantageous, there are of course many situations of inherited genetic disorders where lifelong correction or compensation is necessary. Although issues relating to the immunogenicity of adenoviral vectors are important in this context, another significant issue is that transgenes delivered by adenoviral vec-

tors are almost always not integrated into the host-cell DNA and are thus lost from subsequent generations of cells. In contrast, one of the chief advantages of retrovirus and adeno-associated virus (AAV) vectors is their ability to integrate transgene DNA, thus allowing for long-term gene expression. Use of retroviruses in vivo has been limited by issues of low titer production and complement-mediated inactivation of the vector. AAV vectors have also been limited by low titer production, the presence of contaminating helper adenovirus, and relatively small packaging capacity, although some of these problems have recently been addressed (Ferrari et al. 1997). When comparing the individual features of adenoviral and retroviral vectors, it becomes apparent that the relative strengths of one vector complements the weaknesses of the other. Thus, we developed a chimeric system to take advantage of the positive attributes of both, hypothesizing that such a system would potentially allow for high efficiency in vivo gene delivery coupled with integration of the delivered transgene and long-term gene expression (Feng et al. 1997). In addition to exploiting the individual attributes of the virus vectors, this approach seeks to capitalize on the knowledge that in vivo use of retroviral producer cells as vectors can achieve a level of gene delivery superior to administration of retroviruses themselves (Culver et al. 1992).

The strategy for the adenovirus/retrovirus chimera system is illustrated in Figure 5. Two adenoviral vectors were constructed, one carrying genes encoding retroviral packaging functions (*gag*, *pol*, and amphotropic *env*) (AdCMVAmpg), the other containing retroviral vector sequences including LTRs, packaging signal, and a green fluorescent protein (GFP) transgene (AdLNCMVGFP). Cells are coinfected with these two vectors, resulting in the transient in situ generation of retroviral producer cells. Progeny of replication-incompetent retroviruses are then released to infect neighbor cells with resultant transgene integration and long-term gene expression. For in vivo application, this system would take advantage of the initial systemic stability and highly efficient gene delivery properties of the adenovirus, followed by in situ generation of retroviruses to avoid the problems of low titer production and systemic instability of this vector, yet exploit its integration capabilities.

This approach was first validated in vitro by infecting cells with either one or both adenoviruses, then using the supernatants generated to infect naïve cells. The naïve cells incubated in the supernatant generated from the cells infected with the two viruses expressed GFP for at least 20 days, whereas those exposed to the supernatant from the cells infected with AdLNCMVGFP alone did not express any GFP. These results were

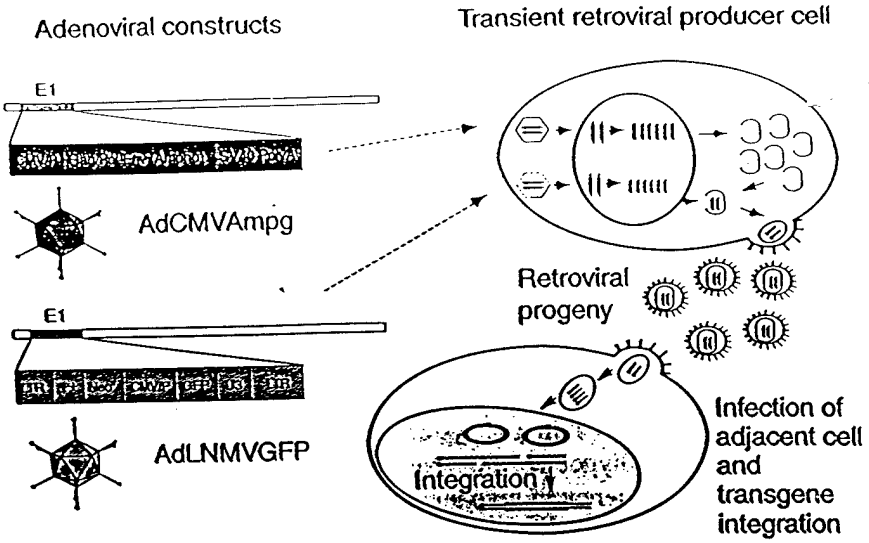

Figure 5. Schema illustrating the adenovirus/retrovirus chimera concept.

therefore consistent with retrovirus production from the initial coinfection with the adenoviral vectors. Subsequently, it was shown by Southern blot that those cells expressing GFP long-term had integration of proviral sequence into their chromosomal DNA.

To validate this approach in vivo, a murine ovarian carcinoma model was used. In the first instance, SKOV3.ip1 cells were infected in vitro with either AdLNCMVGFP alone (as a control) or AdCMVGFP plus AdCMVAmpg, then mixed 1:4 with naïve cells and implanted into nude mice. When sacrificed 20 days later, the tumors from the mice receiving cells infected with both vectors had large sheets of GFP-expressing cells comprising greater than 25% of the tumor mass and therefore consistent with lateral retroviral infection. In contrast, the tumors from the control mice had only rare isolated GFP-expressing cells. In a more stringent experiment, tumors were first established in nude mice by intraperitoneal injection of SKOV3.ip1 cells, and then the mice were then injected with either AdLNCMVGFP alone or AdCMVGFP plus AdCMVAmpg. Only those tumors from mice injected with both vectors expressed GFP when sacrificed. The adenovirus/retrovirus approach combines the favorable attributes of two separate vectors into the one vector system, thus illustrating the key concept of using chimerism to functionally combine the attributes of distinct vectors.

CONCLUSION

The studies discussed in this chapter illustrate the substantial progress being made toward one of the key criteria for successful gene therapy: specificity of delivery to appropriate target cells. Recent advances, such as those outlined in this chapter, reflect the commitment of the field to important basic vectorology issues, and should provide a secure foundation from which to extend the application of gene therapy strategies to human disease.

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Ectodomain of Coxsackievirus and Adenovirus Receptor Genetically Fused to Epidermal Growth Factor Mediates Adenovirus Targeting to Epidermal Growth Factor Receptor-Positive Cells

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Human adenovirus (Ad) is extensively used for a variety of gene therapy applications. However, the utility of Ad vectors is limited due to the low efficiency of Ad-mediated gene transfer to target cells expressing marginal levels of the Ad fiber receptor. Therefore, the present generation of Ad vectors could potentially be improved by modification of Ad tropism to target the virus to specific organs and tissues. The fact that coxsackievirus and adenovirus receptor (CAR) does not play any role in virus internalization, but functions merely as the virus attachment site, suggests that the extracellular part of CAR might be utilized to block the receptor recognition site on the Ad fiber knob domain. We proposed to design bispecific fusion proteins formed by a recombinant soluble form of truncated CAR (sCAR) and a targeting ligand. In this study, we derived sCAR genetically fused with human epidermal growth factor (EGF) and investigated its ability to target Ad infection to the EGF receptor (EGFR) overexpressed on cancer cell lines. We have demonstrated that sCAR-EGF protein is capable of binding to Ad virions and directing them to EGFR, thereby achieving targeted delivery of reporter gene. These results show that sCAR-EGF protein possesses the ability to effectively retarget Ad via a non-CAR pathway, with enhancement of gene transfer efficiency.

Adenovirus (Ad) represents a large family of nonenveloped viruses containing a double-stranded DNA genome of approximately 36 kb (19, 32). Human Ad includes 49 known viral serotypes grouped into six distinct subgroups, A to F. Ad has been widely used as a vector for both in vitro and in vivo gene delivery, largely because of its relatively high infection efficiency in a variety of cell types and tissues (37, 54). However, the broad tropism of the virus represents a drawback when gene delivery to a specific tissue is needed. Most of the studies on the mechanism of Ad infection have concluded that the host range of Ad seems to be dependent to a large extent on the interaction with primary binding receptor. In this regard, the initial steps of Ad infection involve at least two sequential virus-cell interactions, each mediated by a specific capsid protein of the viral particle. Ad infection is initiated by the formation of the complexes between globular knob domain of the fiber protein and a host cell primary receptor (12, 25, 39). Three putative Ad fiber receptors have been described to date. A fiber receptor for Ad of groups A, C, D, E, and F has been identified as the coxsackievirus group B and Ad receptor (CAR) (3, 34, 42). In addition to CAR, the major histocompatibility complex class I (MHC-I) α2 subunit was also proposed as a cell receptor for subgroup C (18). However, CAR has been suggested to mediate high-affinity binding to Ad fiber, while the MHC-I α2 subunit has been hypothesized to facilitate Ad attachment and permissivity to cells with little or no CAR expression (7). It was shown recently that Ad serotype 37 (Ad37) of subgroup D uses α(2→3)-linked sialic acid saccharides on glycoproteins as the cellular receptor moiety instead of CAR or MHC-I α2 (1). After binding to the fiber receptor, penton base interaction with $\alpha_v\beta$ integrins facilitates internalization via receptor-mediated endocytosis (14, 27, 50). These data suggest that, being expressed in a wide range of human and murine cell types (42), CAR may serve as a primary cellular receptor for the majority of representatives of known Ad serotypes. CAR is an integral membrane protein of unknown cellular function consisting of two extracellular immunoglobulin (Ig) superfamily domains, a single membrane-spanning region, and one carboxy-terminal cytoplasmic domain (3, 4, 42). According to the recent crystallography study of Ad12 fiber knob domain complexed with CAR amino-terminal Ig1 domain, three CAR monomers bind per knob trimer, indicating the location of CAR-binding sites on the knob (5). Furthermore, it was demonstrated that the extracellular domain of CAR is sufficient to allow virus attachment and infection (11, 34), while the transmembrane and intracellular regions appear to be dispensable for these functions (44).

Although Ad vectors can infect most cells, a few cell types including endothelial (23), lung epithelial (38), smooth muscle (33), neural (22), and T (2) cells are poorly infected by Ad apparently due to the scarcity of an appropriate cell surface receptor. The limitations associated with broad native tropism of Ad and low-efficiency gene delivery to Ad receptor-deficient cells could be solved by redirecting the binding of virus to a specific cellular receptor present at sufficient magnitude on target cells. Several strategies are currently being considered to redirect the Ad in order to confer targeting capability or to enhance vector infectivity. In this regard, the incorporation of a targeting peptide ligand by genetic virion modifications offers a rational approach (8, 35, 51, 53) but has several limitations; the capacity for peptide substitution or addition to capsid proteins is size limited, and such modifications can often interfere

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with correct protein folding and consequent virus assembly (53). On the other hand, the technical achievement of Ad retargeting via bispecific molecules has been approached by a variety of methods. Chemically conjugated bispecific antibodies have been used, with viral linkage accomplished via a peptide epitope incorporated in the penton base (49, 52) or via specific recognition for the knob domain of the fiber protein (9, 13, 29, 41). Further refinement of the strategy of the retargeting complexes has been achieved by the engineering of recombinant proteins consisting of an antiknob single chain fragment variable (scFv) of antibody fused with human epidermal growth factor (EGF) (45) or anti-EGF receptor (EGFR) scFv (16). Recombinant molecules such as these may offer advantages for Ad retargeting, since use of the chemical conjugation method, as well as antibody-containing molecules, increases the difficulties of producing such retargeting complexes, making this approach relatively complex and expensive to develop. Consequently, a simple and efficient method of targeting Ad infection to specific cells would be of great utility for significantly improving present Ad vectors for gene therapy.

In this study, we have developed a targeting approach based on achieving a linkage to the vector particle through a soluble form of its own cellular receptor. Specifically, we have derived a bispecific targeting protein consisting of the ectodomain of CAR in fusion with human EGF. This recombinant fusion protein has the ability to effectively retarget the vector via non-CAR pathways, with enhancement of gene transfer efficiency. In addition, this approach may allow the derivation of different fusion proteins that are capable of Ad retargeting to other cellular receptors by a simple substitution of targeting ligand.

MATERIALS AND METHODS

Cells. The 293 human kidney cell line transformed with Ad5 DNA was purchased from Microbix (Toronto, Ontario, Canada). The human ovarian carcinoma cell line SKOV3.ip1 was obtained from Janet Price (M. D. Anderson Cancer Center, Houston, Tex.). The human epidermoid carcinoma cell line (A-431), human squamous carcinoma (SCC-4) cells, and human mammary gland (MDA-MB-453) cells were from the American Type Culture Collection (Manassas, Va.). All cell lines were grown at 37°C in media recommended by the suppliers in a humidified atmosphere of 5% CO_2.

Enzymes. Restriction endonucleases, Klenow enzyme, T4 DNA ligase, and proteinase K were from either New England Biolabs (Beverly, Mass.) or Boehringer Mannheim (Indianapolis, Ind.).

Antibodies. Murine monoclonal antibody (MAb) 4D2 to the tail domain of Ad5 fiber protein (20) and murine polyclonal serum to baculovirus-produced human soluble CAR (sCAR) protein were generated at the University of Alabama at Birmingham Hybridoma Core Facility. Murine MAb 425 to human EGFR was a generous gift from Zenon Steplewski (Thomas Jefferson University, Philadelphia, Pa.).

Viruses. A recombinant Ad5 vector, AdCMVLuc, containing a firefly luciferase-expressing cassette in place of the E1 region of the Ad genome, was obtained from R. D. Gerard (University of Texas Southwestern Medical Center, Dallas). Ad was propagated on 293 cells and purified by centrifugation in CsCl gradients by a standard protocol. Virus particle titer was determined spectrophotometrically by the method of Maizel et al. (26), using a conversion factor of 1.1×10^{12} viral particles per absorbance unit at 260 nm. To determine the titer of infectious viral particles, the plaque assay on 293 cells was performed by the method of Mittereder et al. (30). Radiolabeled Ad was made by adding 50 μCi of [*methyl*-^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, N.J.) per ml to the medium of infected cells at 20 h postinfection at a multiplicity of infection (MOI) of 2.5 PFU/cell. The infected cells were then harvested at 50 h postinfection, and the virus was purified as described above. The activity of the labeled virus was approximately 10^{-4} cpm/virus particle.

Indirect immunofluorescence. Confluent cells were released with EDTA and resuspended in HEPES-buffered saline (20 mM HEPES [pH 7.4], 1% bovine serum albumin (BSA)] at 2×10^6 cells/ml. Cells (2×10^5) were incubated with either MAb 425 (5 μg/ml) or murine anti-CAR serum (1:250) for 1 h at 4°C. Either an isotype-matched IgG (4D2) or normal murine serum was used as a negative control. Cells were then washed with buffer and incubated with secondary goat anti-mouse IgG labeled with fluorescein isothiocyanate (Jackson Laboratories, West Grove, Pa.) at a concentration 5 μg/ml for 1 h at 4°C. After washing, 10^4 cells per sample were analyzed by flow cytometry performed at the University of Alabama at Birmingham FACS Core Facility. Data were expressed as the geometric mean fluorescence intensity of the entire gated population. The positive population cells was determined by gating the right-hand tail of the distribution of the negative control sample for each cell line at 1%. This gate setting was then used to determine the percentage of CAR- or EGFR-positive cells in each cell line.

Construction of recombinant plasmids. To introduce the six-His purification tag into the carboxy terminus of sCAR, oligonucleotides 5′GAT CCC CCC GAT ATC ACC ATC ACC ATC ACT AAT AAA 3′ and 5′ GAT CTT TAT TAG TGA TGG TGA TGG TGA TAT CGG GGG 3′ were designed to form DNA duplex coding for histidines followed by two in-frame stop codons. In addition, the generated DNA duplex contained *Bam*HI-compatible cohesive ends and an *Eco*RV restriction site designed to fuse the CAR open reading frame with six-His coding sequence. The oligonucleotide duplex was cloned into *Bam*HI-digested pQBI-AdCMV5 (Quantum Biotechnologies Inc., Montreal, Quebec, Canada). Plasmid clones were then sequenced in the region of the insert, and the plasmid containing the duplex in the correct orientation was designated pQBI-AdCMV5.6h. To generate a gene encoding the extracellular domain of human CAR, PCR was used. Sense primer (5′ AAA CCG CCT ACC TGC AGC CG 3′) complementary to the position 20 of the 5′ untranslated region of human CAR cDNA (3) and antisense primer (5′ GAG CTT TAT TTG AAG GAG GGA CAA CG 3′) complementary to position 767 were designed to fuse the CAR open reading frame with DNA sequence coding for six histidines incorporated in pQBI-AdCMV5.6h. To construct the plasmid containing the sCAR-His_6 gene, a 751-bp PCR fragment was cloned into *Pme*I- and *Eco*RV-digested pQBI-AdCMV5.6h, resulting in plasmid pQBIshCAR.6h. This plasmid encodes 236 amino-terminal amino acids (aa) of an extracellular domain of human CAR, including signal sequence, fused with a carboxy-terminal six-His purification tag. To express human sCAR, the Bac-to-Bac baculovirus expression system (Life Technologies, Grand Island, N.Y.) was used. The recombinant donor plasmid for the generation of baculovirus expressing human sCAR was made as follows. The base donor plasmid pFastBac1 was cleaved with *Acc*65I, and 3′ recessed ends were filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I. The Klenow fragment was heat inactivated, and plasmid was then cleaved with *Pst*I. Plasmid pQBI-shCAR was cleaved with *Bsr*DI and treated with Klenow fragment to remove the 3′ overhang. After inactivation of DNA polymerase, the plasmid was cleaved with *Pst*I and a *Pst*I-*Bsr*DI fragment (808 bp) and gel purified for cloning into *Acc*65I- and *Pst*I-digested pFastBac1. After transformation of *E. coli* strain DH5α (Life Technologies), the resultant plasmid pFBshCAR6h was isolated and used for generation of the recombinant baculovirus genome.

To create the gene for the sCAR-EGF fusion protein, the DNA sequence coding for a short flexible linker and human EGF was amplified from plasmid pBsF5slEGF (unpublished data) using the primers 5′ CCC ATT GGC CAT CAG CCT CCG CAT C 3′ and 5′ GCC CCC GCT CGA GGT CGA CGG TAT C 3′. The PCR-derived DNA fragment contained a unique 5′ *Msc*I site and 3′ *Sal*I site introduced into the molecule to facilitate subsequent cloning. The PCR product was cleaved with *Msc*I and *Sal*I, and a 282-bp DNA fragment was gel purified for further cloning. To construct the plasmid containing the gene coding for sCAR-EGF, primers 5′ CCC ACG GTC CGG CAG CCA CCA TG 3′ and 5′ TCG GGG GAT CTT TAC ACG TGA TGG TGA TGG 3′ were used to reamplify DNA sequence coding for sCAR-His_6 using pFBshCAR as the template. The PCR product cleaved at the *Rsr*II restriction site introduced into the 5′ end of the DNA molecule was then cloned into *Rsr*II- and *Stu*I-digested pFastBac1, resulting in plasmid pFBshCARfuse. To derive the plasmid containing the recombinant gene encoding sCAR-EGF, the *Msc*I-*Sal*I PCR fragment was ligated with *Pml*I- and *Sal*I-digested pFBshCARfuse. After transformation of *E. coli* DH5α, plasmid clones were sequenced in the region of the insert and the resultant plasmid pFBshCAR-EGF was selected. The constructed plasmid encoding recombinant sCAR fused with EGF and tagged with internal His_6 was then used for generation of the recombinant baculovirus genome.

Expression and purification of six-His-tagged recombinant proteins. Recombinant sCAR-His_6 and sCAR-EGF proteins were expressed in High Five cells (Invitrogen, Carlsbad, Calif.) infected with recombinant baculovirus by the method recommended by the manufacturer. Briefly, High Five cells were maintained in suspension culture and infected with recombinant baculovirus at an MOI of 10 PFU/cell. The cell suspension was harvested 72 to 96 h postinfection, and cells were pelleted by centrifugation. Cleared supernatant medium was concentrated 10-fold and dialyzed against phosphate-buffered saline (PBS; 0.01 M PBS [pH 7.4], 138 mM NaCl, 2.7 mM KCl) using a Hemoflow capillary dialyzer (Fresenius Medical Care AG, Bad Homburg vor der Höhe, Germany). Recombinant proteins were then purified by immobilized metal ion affinity chromatography on Ni-nitrilotriacetic acid (NTA)-Sepharose (Qiagen, Valencia, Calif.) as recommended by the manufacturer. Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, Calif.) with bovine gamma globulin as the standard.

ELISA. Solid-phase binding enzyme-linked immunosorbent assay (ELISA) was performed by a method previously described (8). Either purified sCAR-His_6 or sCAR-EGF was diluted in 50 mM carbonate-bicarbonate buffer (pH 9.6) to a concentration of 8.0 pmol/ml, and 100-μl aliquots were added to wells of a 96-well Nunc-Maxisorp ELISA plate. Plates were incubated overnight at 4°C and then blocked for 2 h at room temperature by the addition of 200 μl of blocking

buffer (0.01 M PBS [pH 7.4], 138 mM NaCl, 2.7 mM KCl, 0.05% Tween 20, 2% BSA) to each well. Wells were then washed three times with washing buffer (0.01 M PBS [pH 7.4], 138 mM NaCl, 2.7 mM KCl, 0.05% Tween 20). Purified Ad5 fiber protein diluted in binding buffer (0.01 M PBS [pH 7.4], 138 mM NaCl, 2.7 mM KCl, 0.05% Tween 20, 0.5% BSA) to concentrations ranging from 0.46 to 11 ng/ml was added to the wells in 100-μl aliquots. After 1 h of incubation at room temperature, the wells were washed three times and bound fiber was detected by incubation with 1:1,000 dilution of MAb 4D2. Following incubation at room temperature for 1 h, the wells were washed again and incubated with a 1:10,000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma, St. Louis, Mo.) for 45 min. The wells were then washed four times, and the plate was developed with *p*-nitrophenyl phosphate (Sigma) as recommended by the manufacturer. Plates were read in a microtiter plate reader set at 405 nm; results are presented as mean ± standard deviation (SD).

Competitive inhibition analysis. The ability of sCAR-EGF to bind to EGFR was evaluated by competition analysis of radiolabeled EGF binding to EGFR-positive cells in the presence of increasing concentrations of sCAR-EGF. Briefly, A-431 cells were harvested and resuspended in binding buffer (PBS [pH 7.2], 0.1% BSA) at 5×10^6 cells/ml. The cells were aliquoted (100 μl per sample) in triplicate into polystyrene tubes followed by addition of 10-fold dilutions (~1 pM to 20 μM) of unlabeled human EGF (Pepro Tech, Inc., Rocky Hill, N.J.), sCAR-EGF, or sCAR-His_6 used as a negative control. [^{125}I]EGF (100 μl; ~0.1 nM; Amersham Pharmacia Biotech) was then added, and the cells were incubated at 4°C for 90 min. The cells were then rinsed once with ice-cold buffer and centrifuged at 1,700 × *g* for 10 min, and the supernatant was removed. The cells were then counted in a gamma counter to determine the amount of bound radioactivity.

Radiolabeled Ad binding assay. Binding of ^{3}H-labeled Ad to 293, MDA-MB-453, A-431, SCC-4, or SKOV3.ip1 cells was assayed as follows. Three microliters of ^{3}H-labeled AdCMVLuc (~5.6×10^5 cpm) was preincubated with either sCAR-His_6 or sCAR-EGF for 1 h at room temperature. Confluent cells were released with EDTA, washed once with PBS, pelleted, and resuspended to a final concentration of 10^7 cells/ml in binding medium (Dulbecco modified Eagle medium-Ham's F12, [DMEM-F12], 20 mM HEPES, 0.5% BSA). Then 100-μl aliquots of the cells were transferred to 5-ml test tubes and kept at 4°C. Virus mixtures were then diluted with binding medium to 100 μl, and 25-μl aliquots were added to cell samples and incubated at 4°C with shaking to allow binding. After a 1-h incubation, the cells were washed with 4 ml of binding buffer and centrifuged. Supernatant containing unbound virus was aspirated and cell pellets were solubilized in EcoLume scintillation cocktail (ICN Biomedicals, Costa Mesa, Calif.); then cell-associated radioactivity was measured in a liquid scintillation analyzer (Packard, Downers Grove, Ill.).

Purification of Ad/sCAR-EGF complexes by gel filtration. A column of Sephadex G-100 (Sigma) was prepared with a bed volume of 10 ml (size exclusion volume, 3.6 ml) and washed with equilibration buffer (PBS containing 0.5% BSA). AdCMVLuc preincubated in the presence or absence 0.4 μg of sCAR-EGF for 30 min was diluted to 2 ml with equilibration buffer, and a 1-ml sample was loaded onto the column at gravity flow. Once the sample was loaded onto the column, an additional 1.5 ml of equilibration buffer was applied. The fraction containing the high-molecular-weight Ad or Ad/sCAR-EGF complexes was eluted with 3.3 ml of equilibration buffer solution. The eluted fraction was used immediately for the gene transfer assay.

Ad-mediated gene transfer assay. Ad-mediated infection experiments using cell lines were performed as follows. Cell monolayers grown in a 24-well plate (5×10^5 cells/well) were washed with PBS. AdCMVLuc (5×10^6 PFU) was preincubated with either sCAR-His_6 or sCAR-EGF for 1 h at room temperature. Virus mixtures were then diluted with DMEM-F12 (Mediatech, Herndon, Va.) containing 2% fetal bovine serum to a final concentration of 5×10^6 PFU/ml, and 200-μl aliquots were added to wells (at an MOI of 2 PFU/cell) to allow internalization of AdCMVLuc for 45 min at room temperature. Virus complexes were then aspirated, the cells were washed with PBS, and 1 ml of complete growth medium containing 10% fetal bovine serum and 2 mM glutamine was added to each well. The cells were incubated at 37°C to allow expression of the luciferase gene. Forty hours after the addition of virus, cells were lysed with 250 μl of lysis buffer and analyzed for luciferase expression. Luciferase activity in the cell lysates was analyzed by using the Promega (Madison, Wis.) luciferase assay system and a Berthold (Gaithersburg, Md.) luminometer.

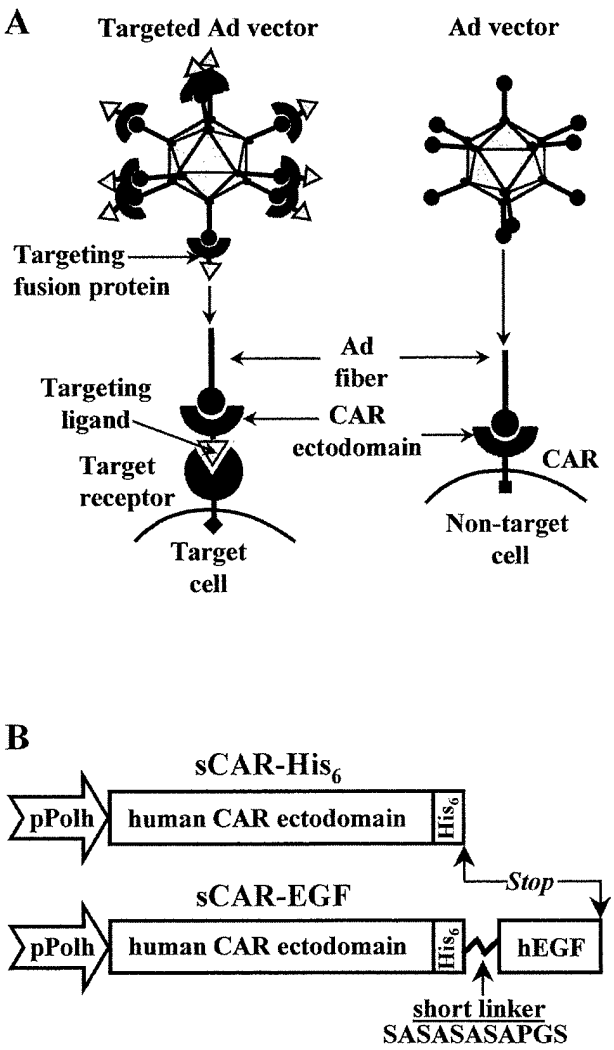

FIG. 1. (A) Utilization of sCAR-ligand fusion proteins for receptor-specific targeting of Ad vectors. Ad vectors normally achieve cell binding via interaction between the knob domain of viral fiber protein with CAR. To redirect an Ad vector to an alternative cell surface receptor, a genetically engineered targeting fusion protein consisting of the CAR ectodomain fused to a receptor-specific targeting ligand was used. By virtue of its dual binding capacity, this complex serves as a bridge between an Ad virion and a cell-specific receptor molecule, thereby providing novel cell-binding capacity to the virion. (B) Construction of sCAR fusion proteins. The gene coding for either human sCAR-His_6 or sCAR-EGF was constructed in a baculovirus expression vector. Expression is driven from the polyhedrin promoter (pPolh). A His_6 tag was introduced for purification purposes into the carboxy terminus of the extracellular CAR domain (236 aa). To construct sCAR-EGF protein, human EGF (53 aa) was fused with the CAR ectodomain by a flexible linker (SASASASAPGS) and tagged with His_6. See Materials and Methods for details of construction.

RESULTS

Design and generation of sCAR-ligand protein. As was recently demonstrated, Ad of subgroups A, C, D, E, and F use CAR as a cellular fiber receptor. It was also shown that sCAR bound to representatives of all Ad subgroups except subgroup B (34). To apply this finding to Ad retargeting, we proposed to design protein molecules consisting of the extracellular domain of CAR in fusion with a targeting ligand (Fig. 1A). Our goal was to generate a bispecific CAR-ligand molecule that could block the cell-binding domain of the fiber knob as well as target the Ad vector to a novel cellular receptor present at a sufficient level on target cells. Therefore, the infection of cells by this virus complex would not be dependent on the presence of CAR on a target cell membrane. The targeting ligand that we chose to use was EGF because it is well established that the EGFR is overexpressed on a variety of cancer cells (46).

To express the sCAR-EGF fusion protein, we designed a gene sequence coding for the ectodomain of human CAR, six histidines, a short flexible linker, and human EGF (Fig. 1B). The sequence encoding the internal six-His tag was incorporated into the recombinant CAR fusion gene in order to facil-

itate downstream purification of the product. The gene coding for sCAR-His_6 was created to express the relevant control protein (Fig. 1B). To produce sCAR-EGF and sCAR-His_6, recombinant baculoviruses containing the genes of interest were created and used to infect insect cells. Infection of High Five insect cells with recombinant baculoviruses resulted in a high level of sCAR-His_6 as well as sCAR-EGF protein expression in a secreted soluble form. Baculovirus-expressed sCAR-His_6 and sCAR-EGF proteins were recovered from the infected cell culture media by means of Ni-NTA affinity chromatography and were then analyzed for purity by gel electrophoresis.

Analysis of sCAR-EGF protein. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of both sCAR-His_6 and sCAR-EGF (Fig. 2A) shows that purified proteins have molecular masses close to expected of 24.9 and 31.9 kDa, respectively. Thus, by using the baculovirus expression system and Ni-NTA affinity purification, we were able to obtain preparative amounts of homogeneously purified sCAR-EGF fusion protein for subsequent analysis.

We first chose to characterize the sCAR-EGF fusion protein with respect to its ability to bind Ad fiber knob. Therefore, we used it in an ELISA (Fig. 2B) with purified Ad5 fiber expressed in insect cells (8). This assay showed that fiber protein efficiently bound to immobilized sCAR-EGF in a wide range of concentrations. Compared to the sCAR-His_6 protein used as a control, the fiber-binding affinity of sCAR-EGF fusion protein was slightly lower, most probably due to changes in the molecular conformation of the CAR ectodomain. Based on the obtained result, whereby generated sCAR-EGF fusion protein is able to efficiently interact with Ad fiber knob, we hypothesized that the affinity of this interaction is sufficient to block the cell-binding site on the knob domain in order to block viral infection.

To evaluate the ability of sCAR-EGF to bind to EGFR, we performed competition analysis of radiolabeled EGF binding to EGFR-positive A-431 cells in the presence of increasing concentrations of sCAR-EGF. Equimolar concentrations of unlabeled human EGF or sCAR-His_6 protein (negative control) were tested in a parallel. Figure 2C shows that EGF and sCAR-EGF inhibited the binding of [^{125}I]EGF to A-431 EGFR-positive cells (47), while sCAR-His_6 did not. The level of inhibition was similar for each, with EGF having a 50% inhibitory concentration of 24.1 nM, compared to 19.5 nM for sCAR-EGF.

sCAR-EGF inhibits Ad binding and gene transfer to 293 cells. Having established that the sCAR-EGF fusion protein could bind to both Ad fiber and cellular EGFR, we examined whether the formation of bonds between Ad and sCAR protein results in any changes in the capacity of such virus complexes to infect cells. To this end, we first compared the cell-binding efficiencies of Ad/sCAR fusion complexes. To do so, we preincubated ^{3}H-radiolabeled Ad with either sCAR-His_6 or sCAR-EGF and then used the formed complexes in a cell-binding assay. 293 human kidney cells were selected for this analysis because they express high levels of CAR and readily support Ad infection (8). The binding assay was performed under conditions (4°C) allowing the viruses to bind the cells but preventing virus internalization. As shown in Fig. 3A, the cell-binding capacity of both Ad/sCAR-His_6 and Ad/sCAR-EGF complexes was less than that of Ad alone and dependent on the sCAR-fusion protein dose; 94 pmol of both sCAR fusion proteins could block 90% of Ad binding to CAR-positive 293 cells. According to flow cytometry analysis, 293 cells express high levels of CAR and EGFR (data not shown). Because of relatively equivalent expression of CAR and EGFR

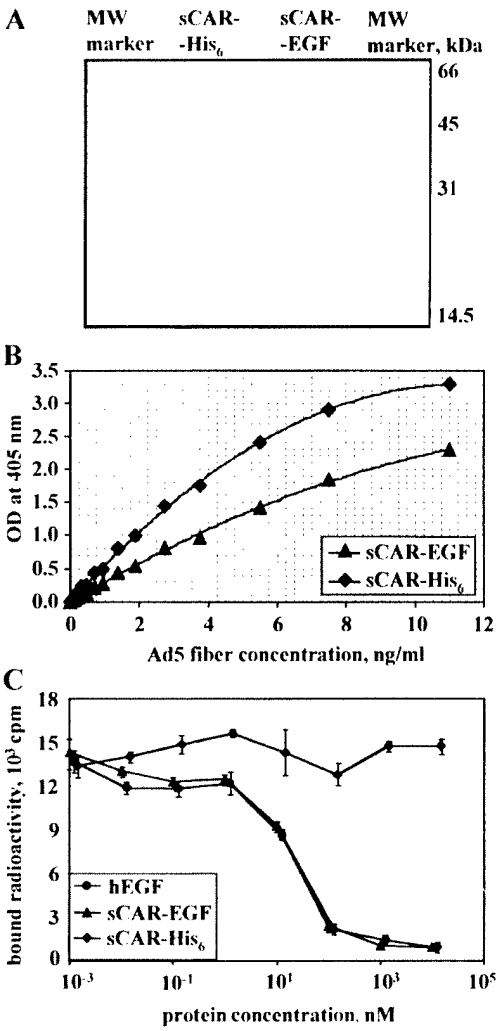

FIG. 2. Characterization of sCAR fusion proteins. (A) Analysis of recombinant sCAR-EGF protein by polyacrylamide gel electrophoresis. Soluble CAR-His_6 and sCAR-EGF six-histidine-tagged proteins expressed in insect cells were purified on a Ni-NTA-Sepharose column and analyzed by electrophoresis on a 12% polyacrylamide gel at denaturing conditions. The bands were visualized by GELCODE blue stain reagent. Numbers on the right indicate molecular masses of marker proteins in kilodaltons. (B) Analysis of interaction between recombinant sCAR-EGF protein and Ad fiber protein by ELISA. Baculovirus-expressed sCAR-His_6 and sCAR-EGF proteins absorbed on an ELISA plate were incubated with various concentrations of purified recombinant Ad5 fiber protein. Bound fiber protein was then detected with antifiber monoclonal antibody 4D2. Each point represents the cumulative mean ± SD of triplicate determinations. Error bars depicting SDs are smaller than the symbols. OD, optical density. (C) Competitive inhibition analysis. The binding of sCAR-EGF to EGFR was quantified by competition analysis of radiolabeled EGF binding to A-431 EGFR-overexpressing cells in the presence of various concentrations of sCAR-EGF. The cells were mixed with 10-fold dilutions (1 pM to 20 μM) of unlabeled human EGF, sCAR-EGF, or sCAR-His_6 (negative control). [^{125}I]EGF (~0.1 nM) was then added, and incubation was continued 4°C. The cells were then rinsed and counted in a gamma counter to determine the amount of bound radioactivity. Each point represents the cumulative mean ± SD of triplicate determinations. Some error bars depicting SDs are smaller than the symbols.

on the cell membrane, binding of Ad/CAR-EGF complexes to 293 cells may occur via any combination of CAR- and EGFR-dependent routes. Therefore, the capacity of sCAR-EGF molecules to block Ad cell binding through a CAR-dependent pathway might be diminished by the alternative ability to mediate binding to EGFR and result in cumulative inhibition of Ad binding and subsequent gene delivery. As can be seen in

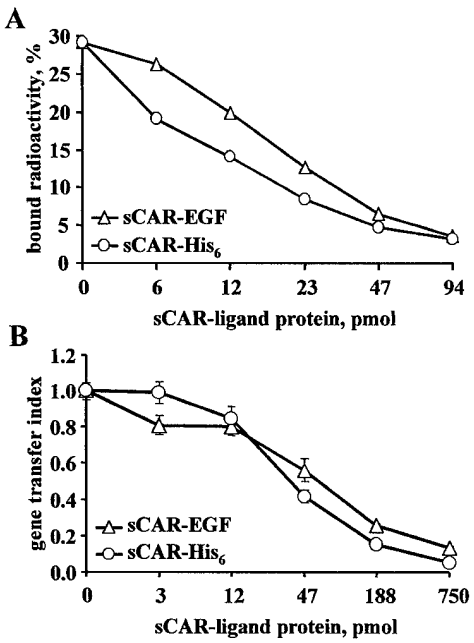

FIG. 3. Functional characterization of sCAR fusion proteins. (A) Inhibition of Ad binding. ^{3}H-labeled Ad was preincubated with different amounts of either sCAR-His_6 or sCAR-EGF, and then ^{3}H-Ad/sCAR-ligand complex samples (10^5 cpm) were mixed with 293 cells (10^6 cells per aliquot) and allowed to bind at 4°C. Cell-bound radioactivities were determined as described in Materials and Methods. Data are presented as the percentage of input ^{3}H-Ad bound after washing and calculated as the cumulative mean ± SD of triplicate determinations. Error bars depicting SDs are smaller than the symbols. (B) Inhibition of Ad-mediated gene transfer. Recombinant Ad vector AdCMVLuc, expressing the firefly luciferase reporter gene, was preincubated with various amounts of either sCAR-His_6 or sCAR-EGF. Monolayers of 293 cells were then exposed to AdCMVLuc/sCAR-ligand complexes and assayed for luciferase activity as described in Materials and Methods. Gene transfer indices were calculated from the ratio of the mean luciferase activity documented in cells infected with either AdCMVLuc/sCAR-EGF or AdCMVLuc/sCAR-His_6 to those treated with AdCMVLuc alone. Each point represents the cumulative mean ± SD of triplicate determinations. Some error bars depicting SDs are smaller than the symbols.

Fig. 3A, sCAR-EGF protein indeed displayed less Ad-blocking ability than the control sCAR-His_6, particularly in the low concentration range.

To evaluate the ability of the derived fusion protein to block Ad infection, we performed an infection inhibition assay. The results showed that sCAR-EGF protein is able to block AdCMVLuc-mediated luciferase gene transfer to 293 cells, demonstrating an inhibition profile similar to that for control sCAR-His_6 protein (Fig. 3B). Therefore, these experiments confirmed the utility of sCAR-EGF to efficiently inhibit Ad binding as well as gene transfer to CAR-positive 293 cells and provided a rationale for further studies.

Analysis of different human cell lines for the expression of CAR and EGFR. Several human cell lines of different origins, including SKOV3.ip1 ovarian carcinoma cells, A-431 epidermoid carcinoma cells, SCC-4 squamous carcinoma cells, and MDA-MB-453 mammary gland cells, were analyzed for cell surface expression of CAR and EGFR by indirect immunofluorescence assay (Fig. 4). The indicated cell lines were chosen based on previously published data on levels of CAR and EGFR expression and varying susceptibility to Ad infection (6, 8). Flow cytometry showed that SCC-4 cells express moderate levels of CAR and rather large amounts of EGFR (Fig. 4). SKOV3.ip1 cells were CAR negative but high EGFR expressors. A431 cells display a low level of CAR expression while

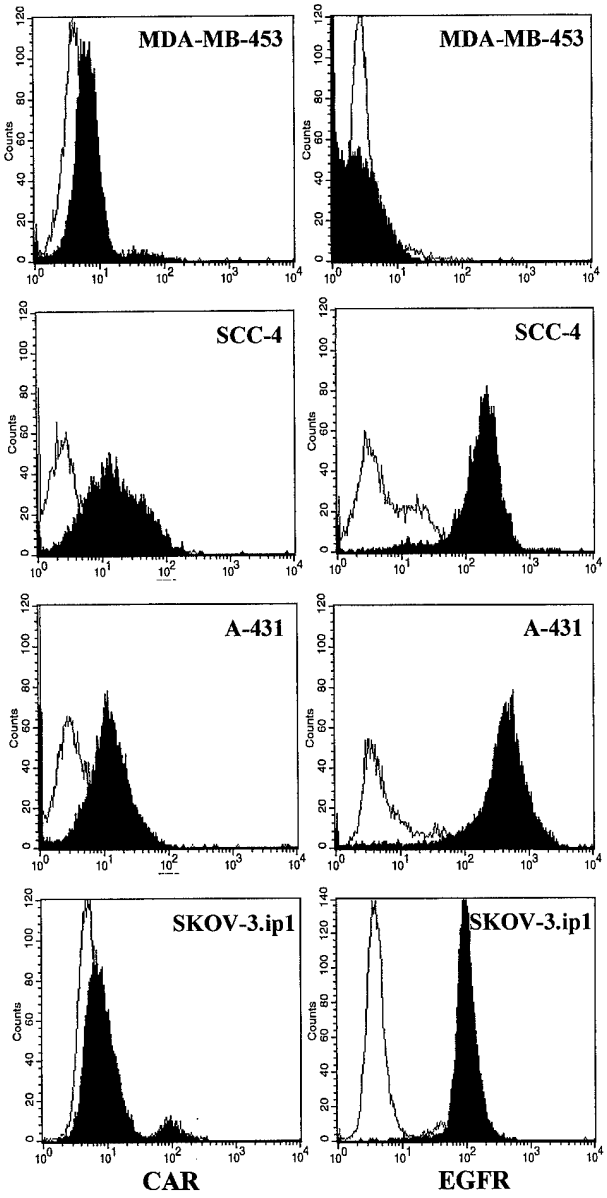

FIG. 4. Relative expression of CAR and EGFR on different human cell lines. Flow cytometric analysis of MDA-MB-453, SCC-4, SKOV3.ip1, or A-431 cells using either murine polyclonal serum to CAR or anti-EGFR MAb was performed as described in Materials and Methods. Positive staining with anti-CAR or anti-EGFR antibody (black) is seen relative to an isotype control (white). A representative of three separate experiments is shown. Flow cytometry assay revealed that SCC-4, SKOV3.ip1, and A-431 cells express high levels of cell surface EGFR but moderate to low levels of CAR. MDA-MB-453 cells demonstrated dramatically lower levels of both CAR and EGFR and were selected as a negative control.

being highest in levels of EGFR. This agrees with previous reports showing that A-431 cells express as many as 3×10^6 EGFR molecules/cell (47). MDA-MB-453 cells, known to be EGFR negative (28), also demonstrated a low level of CAR and were thus selected as a negative control. Flow cytometry data indicate the following order for the tested cell lines with respect to relative expression of EGFR: MDA-MB-453 < SKOV3.ip1 < SCC-4 < A-431. Therefore, for our subsequent

experiments, we established a set of cell lines covering a wide range of EGFR expression.

sCAR-EGF can mediate EGFR-specific cell binding. Having established that sCAR-EGF demonstrates sufficient ability to block Ad infection, we investigated the ability of the Ad/sCAR-EGF complex to infect cells through a CAR-independent pathway. To address this issue, we studied the ability of sCAR-EGF to target Ad binding to EGFR. Retargeting Ad infection through EGFR on cells normally refractory to Ad due to lack of CAR expression on their cell membranes may enhance the level of gene delivery by facilitating Ad binding to EGFR. To investigate this hypothesis, we estimated the capacity of sCAR-EGF fusion protein to mediate binding of ^{3}H-radiolabeled Ad to EGFR-positive cells. To determine the optimum concentration of sCAR-EGF providing maximal Ad binding, ^{3}H-Ad was preincubated with increasing amounts of either sCAR-EGF or sCAR-His$_6$ to allow complex formation. Suspensions of MDA-MB-453, SCC-4, SKOV3.ip1, or A-431 cells were then exposed to either ^{3}H-Ad/sCAR-EGF or ^{3}H-Ad/sCAR-His$_6$ complexes at 4°C to prevent virus internalization. As shown in Fig. 5, preincubation with sCAR-EGF resulted in significant increase of ^{3}H-Ad binding to EGFR-positive SCC-4, SKOV3.ip1, and A-431 cells compared to EGFR-negative MDA-MB-453 cells. Preincubation of Ad in the presence of sCAR-His$_6$ had no effect on the level of binding to EGFR-positive cells compared to Ad alone. Complexing ^{3}H-Ad with increasing amounts of targeting sCAR-EGF fusion protein increased the level of cell-bound radioactivity in a dose-dependent manner. Maximal EGFR-targeted Ad binding occurred with an sCAR-EGF/virus ratio of 12 pmol of sCAR-EGF protein per 6×10^9 viral particles. Increasing the sCAR-EGF/virus ratio further proved inhibitory to binding, presumably because of competition for EGFR binding by excess sCAR-EGF protein. As shown in Fig. 5, calculated binding indices for Ad targeted to EGFR on SKOV3.ip1, SCC-4, and A-431 cells were increased 7-, 8-, and 12-fold, respectively. Bound radioactivities registered in cell samples incubated with EGF-targeted Ad/sCAR-EGF complexes were dependent on sCAR-EGF protein dose and significantly higher than in the case of Ad/sCAR-His$_6$ complexes or Ad alone. These experiments clearly demonstrated that sCAR-EGF protein is capable of inducing changes in the initial steps of virus-cell interaction and suggest that formation of the Ad/sCAR-EGF complexes allows for Ad binding to EGFR.

sCAR-EGF mediates specific Ad binding to cellular EGFR. Since our ultimate goal was the targeting of Ad vectors to EGFR, we conducted a competition assay to prove that sCAR-EGF-mediated virus-cell interactions occurred specifically via EGFR as the alternative cellular receptor. By blocking Ad/sCAR-EGF interaction with a specific competitor, the level of EGFR-dependent binding could be determined. In this regard, analysis of binding of ^{3}H-Ad/sCAR-EGF complexes to the cells was accomplished at 4°C in the presence of either human EGF or anti-EGFR neutralizing MAb capable of blocking the binding to EGFR. To perform this analysis, the optimum sCAR-EGF/virus ratio determined in the binding assay was used to form ^{3}H-Ad/sCAR-EGF complexes prior to the binding to A-431 cells in the presence of increasing concentrations of either human EGF or anti-EGFR MAb. As shown in Fig. 6, binding of ^{3}H-Ad in the absence of competitors was not significantly different from binding in the presence of any tested concentrations of EGF or anti-EGFR MAb. When binding of the ^{3}H-Ad/sCAR-EGF complex was assayed (Fig. 6), the presence of EGF as well as anti-EGFR MAb decreased the level of binding in a dose-dependent manner. Due to significant differences in molar concentrations of the blocking agents used, EGF protein displayed higher blocking efficiency than MAb at low concentrations but similar efficiency at high concentrations tested, blocking 90% of the binding. These results demonstrate that derived sCAR-EGF protein can be effectively used to direct Ad binding via a non-CAR pathway to a novel cellular receptor. Of note, the increased Ad binding efficiency was shown to occur through specific interaction of the sCAR-EGF targeting protein with EGFR.

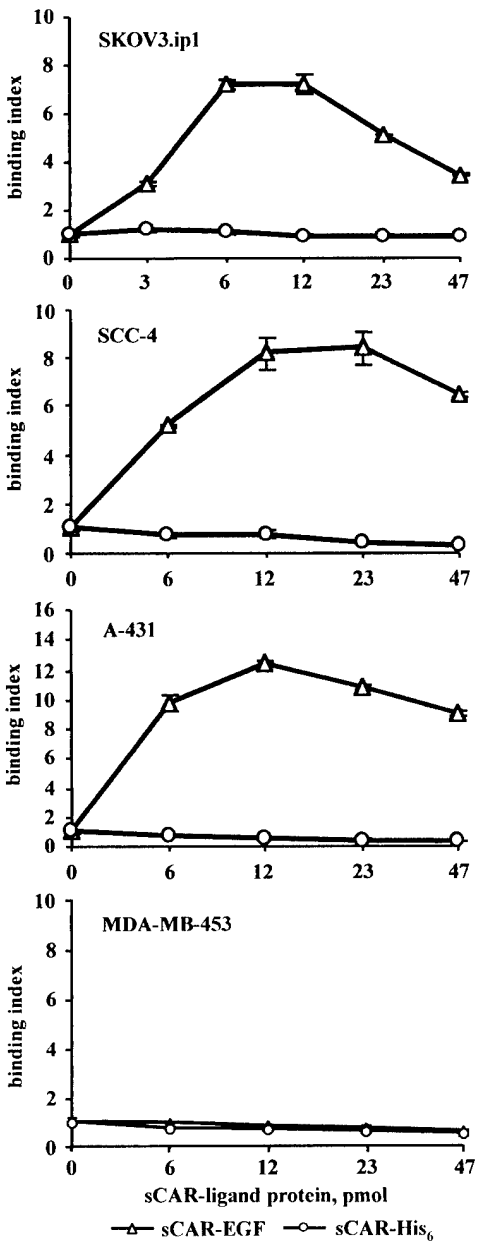

FIG. 5. Comparison of ^{3}H-labeled Ad binding to MDA-MB-453, SCC-4, SKOV3.ip1, and A-431 cells. ^{3}H-labeled Ad was preincubated for 30 min at room temperature with different amounts of sCAR-His$_6$ or sCAR-EGF. ^{3}H-Ad/sCAR-ligand mixtures (10^5 cpm per sample) were then added to cells aliquots (10^6) and allowed to bind for 1 h at 4°C. Bound radioactivity was determined after pelleting the cells by centrifugation. Binding indices were calculated from the ratio of the mean bound radioactivity of ^{3}H-Ad preincubated in presence of sCAR-ligand versus ^{3}H-Ad preincubated in absence of sCAR-ligand protein. Each point represents the cumulative mean ± SD of triplicate determinations. Some error bars depicting SDs are smaller than the symbols.

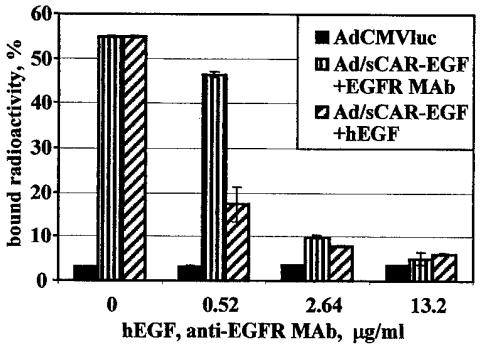

FIG. 6. Specific inhibition of sCAR-EGF-mediated Ad binding. ^{3}H-Ad was preincubated for 30 min at room temperature with 0.4 μg of sCAR-EGF. Human epidermoid carcinoma A-431 cells, overexpressing EGFR, were preincubated for 30 min at 4°C in the presence or absence of either human EGF or anti-EGFR MAb at different concentrations (0.52 to 13.2 μg/ml). ^{3}H-Ad/sCAR-EGF samples (10^5 cpm) were then added and allowed to bind for 1 h at 4°C. Cells were washed by centrifugation, and radioactivities of cell pellets were determined in a beta counter. Data are presented as the percentage of input ^{3}H-Ad bound after washing and calculated as the cumulative mean ± SD of triplicate determinations.

sCAR-EGF mediates enhanced gene transfer to human cancer cells. We used the same strategy to evaluate the ability of sCAR-EGF targeting protein to mediate Ad gene delivery to cultured human cancer cell lines SCC-4, SKOV3.ip1, A-431, and MDA-MB-453. Our previous study showed that these cells are relatively difficult to infect with Ad vectors (6, 8). These findings were corroborated by our flow cytometry data, which showed either modest or low levels of CAR expression. Importantly, rather high levels of EGFR detected in three of these cell lines suggested that low Ad susceptibility due to relative lack of CAR may be overcome by targeting to EGFR present at sufficient magnitude. The sCAR-EGF protein was titered against Ad to ascertain the optimal ratio of targeting protein to virus as measured by improvements in gene transfer. The magnitude of gene expression mediated by the sCAR-EGF-complexed AdCMVLuc was demonstrated on EGFR-positive SCC-4, SKOV3.ip1, and A-431 cells versus EGFR-negative MDA-MB-453 cells. As shown in Fig. 7, compared with AdCMVLuc alone, AdCMVLuc complexed with sCAR-EGF targeting protein (EGFR-targeted Ad) mediated 8-, 10-, and 50-fold enhancements of luciferase expression in SKOV3.ip1, SCC-4, and A-431 cells, respectively. As evidence that the sCAR-EGF promoted gene transfer by an EGFR-specific mechanism, no enhancement was observed in cells exposed to AdCMVLuc complexed with sCAR-His_6 (untargeted Ad). Further, the specificity of sCAR-EGF-mediated Ad targeting was illustrated by the failure of sCAR-EGF to enhance Ad-based gene transfer to EGFR-negative MDA-MB-453 cells. Thus, this set of assays demonstrated that the sCAR-EGF targeting protein enables retargeting of an Ad vector via a CAR-independent pathway, with severalfold enhancement of gene transfer efficiency specifically to EGFR-positive cells.

Efficiency of sCAR-EGF-mediated Ad gene delivery. To further investigate the phenomenon of sCAR-EGF-mediated Ad targeting, we attempted to evaluate the stability of Ad/sCAR-EGF complexes. As we observed previously, exceeding the optimal sCAR-EGF/virus ratio proved to be inhibitory to binding and gene transfer, presumably because of competition for EGFR binding by uncomplexed sCAR-EGF protein. Alternatively, a relative excess of targeting protein is required for optimal formation of Ad/sCAR-EGF complexes. To address this issue, we purified Ad/sCAR-EGF complexes by gel filtration in order to remove unbound sCAR-EGF protein. Using the optimum sCAR-EGF/virus ratio determined with A-431 cells, gene transfer mediated by purified versus unpurified EGFR-targeted AdCMVLuc was compared among the panel of EGFR-positive cell lines. It was shown that EGFR-targeted AdCMVLuc significantly enhanced gene transfer to tested cells (data not shown). Purified Ad/sCAR-EGF complexes were somewhat less effective than unpurified EGFR-targeted virus, demonstrating 50% less efficient gene transfer. In con-

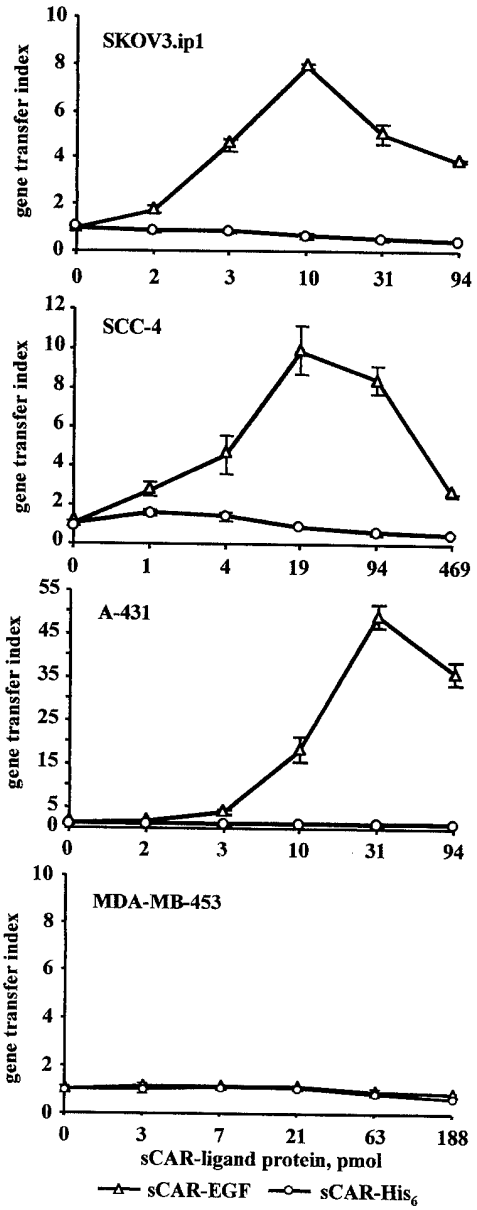

FIG. 7. Characterization of sCAR-EGF-mediated Ad gene transfer to EGFR-positive cell lines. AdCMVLuc was preincubated with various amounts of either sCAR-EGF targeting protein, or sCAR-His_6 as a control, prior to incubation with cells. Then monolayers of MDA-MB-453, SCC-4, SKOV3.ip1, or A-431 cells were exposed to Ad/sCAR-ligand complexes mixtures at 2 PFU/cell. Targeting index is defined as the ratio between mean luciferase activity for Ad preincubated in the presence of sCAR-ligand versus Ad preincubated in absence of sCAR-ligand protein. Each point represents the cumulative mean ± SD of triplicate determinations. Some error bars depicting SDs are smaller than the symbols.

trast, gene transfer mediated by AdCMVLuc was barely affected by purification, indicating that there was no significant loss of Ad during the purification procedure. Nevertheless, even after purification, Ad-mediated gene transfer efficiency was enhanced when targeted to EGFR compared to control AdCMVLuc in all three cell types examined. Specifically, 3-, 4.5-, and 9-fold increases of luciferase activity were observed in SKOV-3.ip1, SCC-4, and A-431 cells, respectively. The degree of gene transfer enhancement correlates with our flow cytometry and binding data and is likely to be dependent on the CAR/EGFR ratio on the cell surface and on EGFR affinity (29). The fact that purification of formed Ad/sCAR-EGF complexes did not ablate enhanced gene transfer capacity indicates that the sCAR-EGF targeting protein can maintain its association with Ad in the context of vector purification schemes. This relative stability provides an empiric means to derive vector/complex particles optimized with respect to gene transfer applications.

DISCUSSION

The infection spectrum of human Ad is wide with respect to different types of tissues and different age groups of patients (40). However, the present generation of Ad vectors suffer from three important limitations which have prevented the realization of their full potential. One disadvantage is related to vector-induced inflammatory and immune responses precluding readministration of the same vector. Second, Ad can infect a wide range of different cells, which makes it impossible to deliver genes to specific target cell types. The third limitation is the inability of the vector to infect the cells which do not express CAR or express it at low levels. Thus, the primary requirement for the application to gene therapy is the availability of a vector capable of accomplishing effective and selective gene delivery. In this regard, there is increasing evidence that the efficiency of Ad vectors can be limited by a deficiency of appropriate binding and entry mechanisms on the target cell (10, 48, 50, 55). While CAR is widely expressed in vivo (42), low CAR levels (17, 24, 31) or its localization on inaccessible parts of cell (43) can prevent efficient infection by Ad vectors. The requirement for expression of CAR on the target cell represents a hurdle to genetic modification of cells that lack CAR, leading to the strategy of modifying the tropism of Ad vectors. Therefore, the utility of the present generation of Ad vectors for gene therapy may be significantly improved by achieving targeted infection of specific cell types by the virus. To develop a targeted Ad vector, it is necessary both to ablate broad native Ad tropism and to introduce novel tropism, which will allow targeting of certain cell types, including cells that are inherently not sensitive to Ad infection due to a lack of CAR. In this regard, abrogation of Ad fiber binding to its natural receptor is therefore a prerequisite for Ad application particularly in vivo. This goal has been addressed by the development of retargeting complexes which simultaneously recognize the specific capsomer of the viral particle and the targeted cell surface molecule. The technical achievement of Ad retargeting via bispecific molecular complexes has been approached by a variety of methods. In this regard, chemically conjugated bispecific antibodies have been used to recognize a FLAG peptide epitope incorporated in the penton base (49, 52). Such bispecific antibodies with specific recognition for the knob domain of the fiber protein that block the knob-receptor interactions and simultaneously serve to cross-link the virus to alternate cellular receptors were designed (9). Abrogation of Ad native tropism was achieved by the use of the antiknob antibody, or its Fab fragment, conjugated with ligands specific for target cell surface receptors such as folate (9), fibroblast growth factor 2 (13, 36), CD40 (41), and EpCAM antigen (15), as well as antibodies for EGFR (6, 29). These recent advances in Ad vector targeting illustrate the potential utility of employing chemically conjugated bispecific molecular complexes to achieve both an abrogation of Ad native tropism and delivery of Ad vectors to specific cell types. However, the use of chemical conjugates increases production difficulties, making this approach relatively complex and expensive to develop. The strategy that we have developed could be of great utility to avoid at least some of these limitations. Recently, refinements of the strategy of antifiber retargeting complexes have been proposed. The ability to engineer recombinant antibodies has facilitated the production of bispecific antibodies or fusion proteins in bacteria. For example, an "adenobody" consisting of an antiknob scFv and EGF has been derived and used to target Ad to EGFR (45). An analogous approach was successfully used to produce recombinant bispecific scFv comprising both antiknob and anti-EGF scFvs in a eukaryotic expression system (16). Recombinant molecules such as these may indeed offer advantages for Ad retargeting in terms of vector production and validation. However, there is likely to be an immune response directed against virus-encoded antigens and possibly against the scFv molecules.

The novelty of the approach developed in this study is based on the utilization of native Ad-CAR interaction to provide a linkage between a targeting ligand and the viral particle. The affinity of CAR binding to the fiber knob (K_d = 4.75 nM) (21) is comparable with those determined for the highest-binding antiknob scFvs (3 to 12 nM) (45). Based on the crystal structure of Ad12 knob complexed with the Ig1 structural domain of human CAR, three Ig1 monomers bind per knob trimer (5). The predicted CAR/knob binding ratio combined with the high affinity of the interaction may contribute to the efficiency of linkage between Ad particles and sCAR-ligand and consequently to the target receptor. The sCAR-EGF protein is expected to have a very low immunogenic potential in humans because of the endogenous origin of its structural components; therefore, it might provide a high-affinity nonimmunogenic linkage to the viral particle compatible with in vivo gene delivery applications. In addition, carboxy-terminal localization of targeting ligand in the context of sCAR fusion protein mimics the native mechanism of virion binding to its high-affinity receptor.

By complexing Ad with sCAR-EGF, we have blocked the natural cell-binding site of the fiber knob while simultaneously supplying a binding alternative in EGF. We showed that Ad modification with bifunctional sCAR-EGF molecules overcomes the barrier of inefficient gene transfer into specific cancer cell types. As expected, the enhanced binding properties of the EGFR-targeted Ad vector correlated with its ability to infect EGFR-expressing cells from a selected panel of cancer cell lines, as seen in gene transfer experiments. Cell binding of EGFR-targeted Ad could be blocked competitively by preincubation of the cells with either human EGF or anti-EGFR MAb, confirming that the redirected Ad binding was specific to EGFR. The gene transfer efficiency of the Ad when targeted through a non-CAR pathway was markedly improved in all EGFR-positive cell lines examined compared to EGFR-negative cells, suggesting that the efficiency of targeted infection is dependent on EGFR density. Purification of Ad/sCAR-EGF complexes proved our hypothesis that sCAR is capable of providing a high-affinity linkage to the viral particle, compatible with an Ad purification scheme and likely with systemic administration of Ad vectors. Use of the baculovirus system for expressing targeting molecules should overcome potential

problems associated with partial or complete insolubility of some protein ligands and lack of glycosylation upon expression in *E. coli*. Furthermore, the observation that sCAR binds to the fiber knob domains derived from certain serotype representatives from five of the six Ad subgroups (34) suggests that sCAR may be the protein of choice to serve as a universal moiety providing a linkage to the majority of Ad serotypes. In addition, the replacement of EGF with a different ligand should enable targeting of Ad vectors to various cellular receptors. The enhancement of transgene expression that we achieved by means of sCAR-EGF-mediated infection of otherwise refractory cancer cells indicates that this approach has potential for further studies of targeting Ad gene delivery to cancer cells in vivo. In this way, it might be possible to ablate preexisting patterns of Ad infection and establish new ones that will reduce the initial dose of virus, thereby decreasing immediate toxicity and increasing the safety and efficiency of Ad vectors.

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A Conditionally Replicative Adenovirus with Enhanced Infectivity Shows Improved Oncolytic Potency[1]

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ABSTRACT

The absence or the presence of low levels of the Coxsackievirus and adenovirus receptor (CAR) on several tumor types might limit the efficacy of recently proposed tumor-specific or conditionally replicative adenoviruses (CRAds). To address this issue, we used a genetic modification of the fiber knob in the context of an E1A-defective CRAd to allow CAR-independent target cell infection as a means to enhance oncolytic potency. Such infectivity-enhanced CRAd showed higher replication, more efficient infection, and lysis of tumor cells *in vitro*. Of note, the improved antitumor effect of the fiber-modified CRAd could be demonstrated *in vivo*. We conclude that the combination of genomic modification to achieve tumor-selective replication and capsid modification to enhance infectivity yields more potent oncolytic adenoviruses for use in cancer treatment.

INTRODUCTION

CRAds[3] represent a novel and promising approach for treating neoplastic diseases (1, 2). The use of CRAds offers two advantages over conventional gene therapy. First, CRAds have an intrinsic amplification capacity that allows extensive tumor infection, leading to expansive oncolysis by reason of the actual cytopathic effect of the virus. Second, the restriction of viral replication to tumors avoids damage to normal host tissues and improves the therapeutic index. Two strategies have been implemented to achieve specificity: the control of the expression of an essential early viral gene by using tumor-specific promoters (3); and deletions in viral genes encoding proteins that interact with cellular proteins necessary to complete the viral lytic life cycle in normal cells, but not in tumor cells (4). Both CRAd-based strategies have been rapidly translated into clinical trials (5, 6).

However, realization of the full utility of CRAds in cancer therapeutics depends on their ability to infect human tumors. Previous studies on adenovirus-mediated gene delivery to human tumor cells have pointed out the highly variable expression of primary adenoviral receptor, CAR, in neoplastic cells (7, 8), and this variation may curtail the initial infection and lateral propagation of CRAds (9). On the basis of these data, it has been proposed that gene delivery via CAR-independent pathways is required to overcome this aspect of tumor biology (10, 11). We have focused on α_V integrins as enhancers of adenoviral infection according to a previous report on the correlation of the levels of α_V integrins expressed by tumor cells with the efficiency of adenovirus-mediated gene transfer (12). Furthermore, previous studies demonstrate that α_V integrins are aberrantly expressed in several types of cancer (13, 14) and are present in tumor blood vessels of breast cancer and malignant melanoma (15).

Modifications of capsid proteins responsible for adenovirus binding to target cells can alter its tissue tropism. These data favor the incorporation of an Arg-Gly-Asp (RGD) sequence, known to interact with α_V integrins, into the adenovirus fiber to enhance tumor infection. Recently, we developed an approach based on the genetic incorporation of a sequence encoding an RGD peptide into the HI loop of the fiber knob. The addition of RGD-integrin interactions on primary CAR binding confers an expanded tropism to the fiber-modified adenovirus, and this effect has been demonstrated in previous studies (16, 17).

In this study, we combined the fiber knob modification strategy with a CRAd based on a partial deletion of the *E1A* gene, which synthesizes a defective protein unable to bind host cell Rb protein. The selectivity of this mutant adenovirus has been previously demonstrated by Fueyo *et al.* (18) and recently by another group that uses a virus with the same deletion (19). Our results demonstrated that the incorporation of the RGD motif into the fiber of a CRAd enhances its oncolytic potency *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell Lines

A549 human lung adenocarcinoma and LNCaP human prostate cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Both cell lines are defective

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[3] The abbreviations used are: CRAd, conditionally replicative adenovirus; BrdUrd, bromodeoxyuridine; CAR, Coxsackievirus and adenovirus receptor; i.t., intratumoral/intratumorally; Rb, retinoblastoma; FBS, fetal bovine serum; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide.

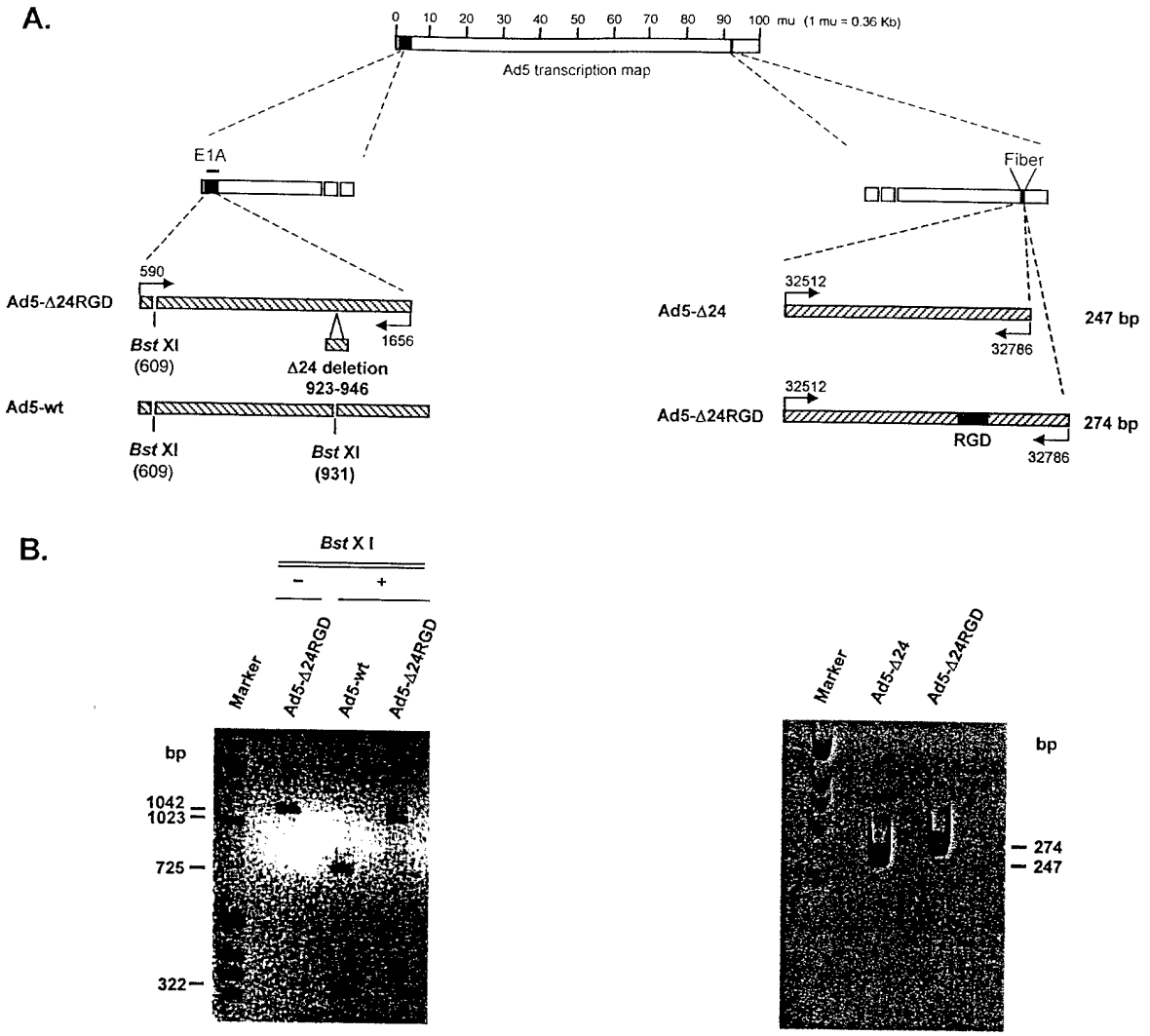

Fig. 1 Analyses of adenoviral DNA. *A*, map of E1A- and fiber-encoding regions of Ad5-Δ24RGD amplified by PCR, showing the 24-bp deletion and the introduced RGD-encoding sequence. *B*, restriction analysis of Ad5-Δ24RGD. The presence of the 24-bp deletion was confirmed by *Bst*XI digestion of the PCR product of the E1A region. The fragments were resolved on a 2% agarose gel, and visualized by UV fluorescence. *Left*, *Marker* (Life Technologies, Inc.), 1-kb DNA ladder. The presence of uncleaved PCR product verified the presence of the deletion. PCR amplification products of the region encoding the fiber from Ad5-Δ24 and Ad5-Δ24RGD were resolved on a 6% acrylamide gel. *Right*, *Marker* (Life Technologies, Inc.), 100-bp DNA ladder. The bigger size (27 bp) of Ad5-Δ24RGD band indicates the presence of the sequence encoding RGD.

in the Rb pathway because of a deficiency in $p16^{INK4}$ (20–22). The cells were cultured in DMEM supplemented with 5% heat-inactivated FBS, 100 I.U./ml penicillin, and 100 μg/ml streptomycin.

Virus Construction

Ad5-Δ24 Mutant. The replication-competent Ad5-Δ24 adenovirus was provided by J. F. (The University of Texas M. D. Anderson Cancer Center, Houston, TX). This virus contains a 24-nucleotide deletion, from Ad5 bp 923 to 946 (both included), corresponding to the amino acid sequence L_{122}TCHEAGF_{129} of the E1A protein known to be necessary for Rb protein binding (23). Details of the tumor-specific replication of this virus are presented elsewhere (18, 19).

RGD Modification of Ad5luc and Ad5-Δ24. Ad5lucRGD is an E1-deleted virus containing the recombinant RGD fiber and expressing the firefly luciferase. This vector was constructed by homologous recombination of the E1 region containing the *luciferase* gene into the plasmid pVK503 that contains the modified fiber (15). A similar procedure was followed to construct the RGD-modified version of Ad5-Δ24. Briefly, an E1 fragment containing the 24-bp deletion was isolated from the plasmid pXC1-Δ24, originally used to construct Ad5-Δ24 (18), and cloned by homologous recombination into the *Cla*I–digested plasmid pVK503 containing the RGD fiber (15). The genome of the new virus was released from the plasmid backbone by digestion with *Pac*I, and the resulting fragment was used to transfect 293 cells to rescue the Ad5-

Δ24RGD. The presence of the RGD motif in Ad5-Δ24RGD and Ad5lucRGD was confirmed by PCR with the fiber primers FiberUp (5′-CAAACGCTGTTGGATTTATG-3′) and FiberDown (5′-GTGTAAGAGGATGTGGCAAAT-3′). The Δ24 deletion was analyzed by PCR with primers E1a-1 (5′-ATTACCGAAGAAATGGCCGC-3′) and E1a-2 (5′-CCATTTAACACGCCATGCA–3′) followed by *Bst*XI digestion.

Virus DNA Replication. A549 cells, cultured in 6-well plates, were infected with Ad5-Δ24 or Ad5-Δ24RGD at a dose of 0.01 viral particles/cell. The cells were maintained in DMEM-5% FBS with 1 μCi/ml BrdUrd (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Attached and detached cells were harvested at 2, 4, 6, and 8 days after infection, and encapsidated viral DNA was purified by the spermine-HCl method (24). The DNA was digested with *Hin*dIII and resolved in 1% agarose gel. The BrdUrd incorporated into the DNA resulting from viral replication was detected by Southwestern blot using mouse anti-BrdUrd IgG (DAKO, Carpinteria, CA) and peroxidase-labeled antimouse antibody (Amersham). The membrane was exposed to Kodak Biomax ML film and developed in an automated processor.

Adenovirus Yield Assay. A549 cells cultured in 6-well plates were infected with 0.01 particle/cell Ad5lucRGD, Ad5-Δ24, or Ad5-Δ24RGD, and maintained in DMEM-5% FBS. After 8 days, cells and media were harvested, and the titer was determined by plaque assay.

Oncolysis Assay. A549 and LNCaP cells cultured by triplicate in 6-well plates were infected with one of the three types of adenovirus at doses of 0.001 or 0.01 viral particles/cell. Eight (A549) and 10 (LNCaP) days after infection, the cells were fixed and stained with crystal violet solution.

***In Vitro* Cytotoxicity Assay (XTT).** A549 and LNCaP cells were seeded and infected in parallel with the ones used for the oncolysis assay described above. Eight and 10 days after infection, cell survival was determined using XTT (Sigma, St. Louis, MO). The number of living cells was calculated from noninfected cells cultured and treated with XTT in the same way as were the experimental groups.

s.c. Tumor Xenograft Model in Nude Mice. Female athymic nu/nu mice (Frederick Cancer Research, Frederick, MD), 8–10 weeks old, were kept under pathogen-free conditions according to the American Association for Accreditation of Laboratory Animal Care guidelines. Eight million A549 cells were xenografted under the skin of each flank in anesthetized mice. When the nodules reached 60–100 mm^3, a single dose of 10^9 viral particles (high-dose experiment; $n = 5$) or 10^7 viral particles (low-dose experiment; $n = 4$) of Ad5lucRGD, Ad5-Δ24, Ad5-Δ24RGD, or PBS was administered i.t. Tumor size was monitored twice a week, and fractional volume was calculated from the formula: (length × width × depth) × 1/2. The mice were euthanized 35 days after the treatment because of the size of the tumors in the control group. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Statistical differences among groups were assessed with Student's *t* tests.

Adenovirus Hexon Immunodetection. The presence of adenovirus hexon in the treated tumor xenografts was assessed by immunofluorescence. A549 tumor sections were treated with goat antihexon (Chemicon Inc., Temecula, CA) and Alexa Fluor 488-labeled donkey antigoat (Molecular Probes, Eugene, OR) antibodies, and were counterstained with Hoechst 33342 (Molecular Probes). The slides were analyzed under a fluorescent microscope (Leitz Orthoplan).

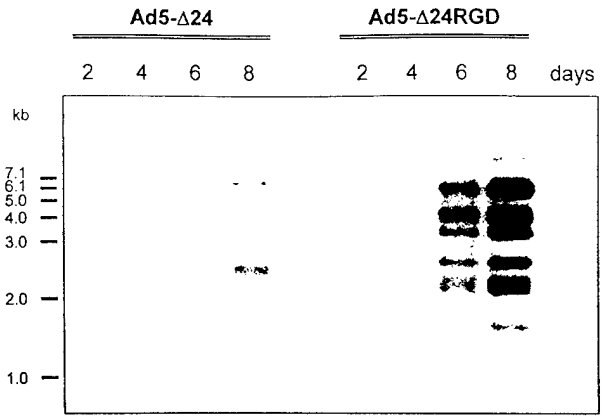

Fig. 2 Propagation efficiency of Ad5-Δ24 *versus* Ad5-Δ24RGD. A549 cells were infected with 0.01 particles/cell Ad5-Δ24 or Ad5-Δ24RGD and incubated in medium containing 1 μCi/ml BrdUrd. At the indicated times after infection, the cells were harvested, and the encapsidated DNA was purified by the spermine-HCl method. Viral DNA from 6 × 10^5 infected cells was digested with *Hin*dIII and electrophoresed, and the resulting fragments were blotted into a membrane that was processed with a mouse anti-BrdUrd antibody. The amount of BrdUrd incorporated into viral DNA indicated that Ad5-Δ24RGD propagation is more efficient than that of Ad5-Δ24.

RESULTS

Propagation Advantage of an RGD-modified CRAd. The Δ24 deletion of E1A and the RGD insertion in the fiber knob were combined into a unique viral genome by homologous recombination, and the resulting Ad5-Δ24RGD was propagated efficiently in A549 cells. The 24-bp deletion in the *E1A* gene and the RGD-encoding sequence in the fiber were verified by PCR (Fig. 1). Of note, no adenoviruses having wild-type E1 or wild-type fiber appeared throughout the propagation of Ad5-Δ24RGD, a finding that confirms the lack of endogenous adenoviral sequences in A549 cells.

After structural confirmation, the replication capacity of Ad5-Δ24RGD and Ad5Δ24 was compared. A549 cells were infected with 0.01 viral particle per cell of each virus and were maintained in medium with BrdUrd throughout the 8-day incubation period. The encapsidated viral DNA was purified on days 2, 4, 6, and 8 postinfection, and the samples were analyzed by Southwestern blot as described in "Materials and Methods." As indicated by the BrdUrd incorporated into replicating viral DNA, Ad5-Δ24RGD propagation was more efficient than that of Ad5-Δ24 (Fig. 2). The Ad5-Δ24RGD DNA can be detected not only sooner (day 6) compared with Ad5-Δ24 DNA (day 8) but in greater amounts. Thus, the infectivity advantage conferred by RGD incorporation into the fiber knob increased adenovirus propagation in target cells.

Increased Viral Yield of Infectivity-enhanced CRAd *in Vitro*. On the basis of the previous experiment, we decided to compare the amount of infectious virus produced by

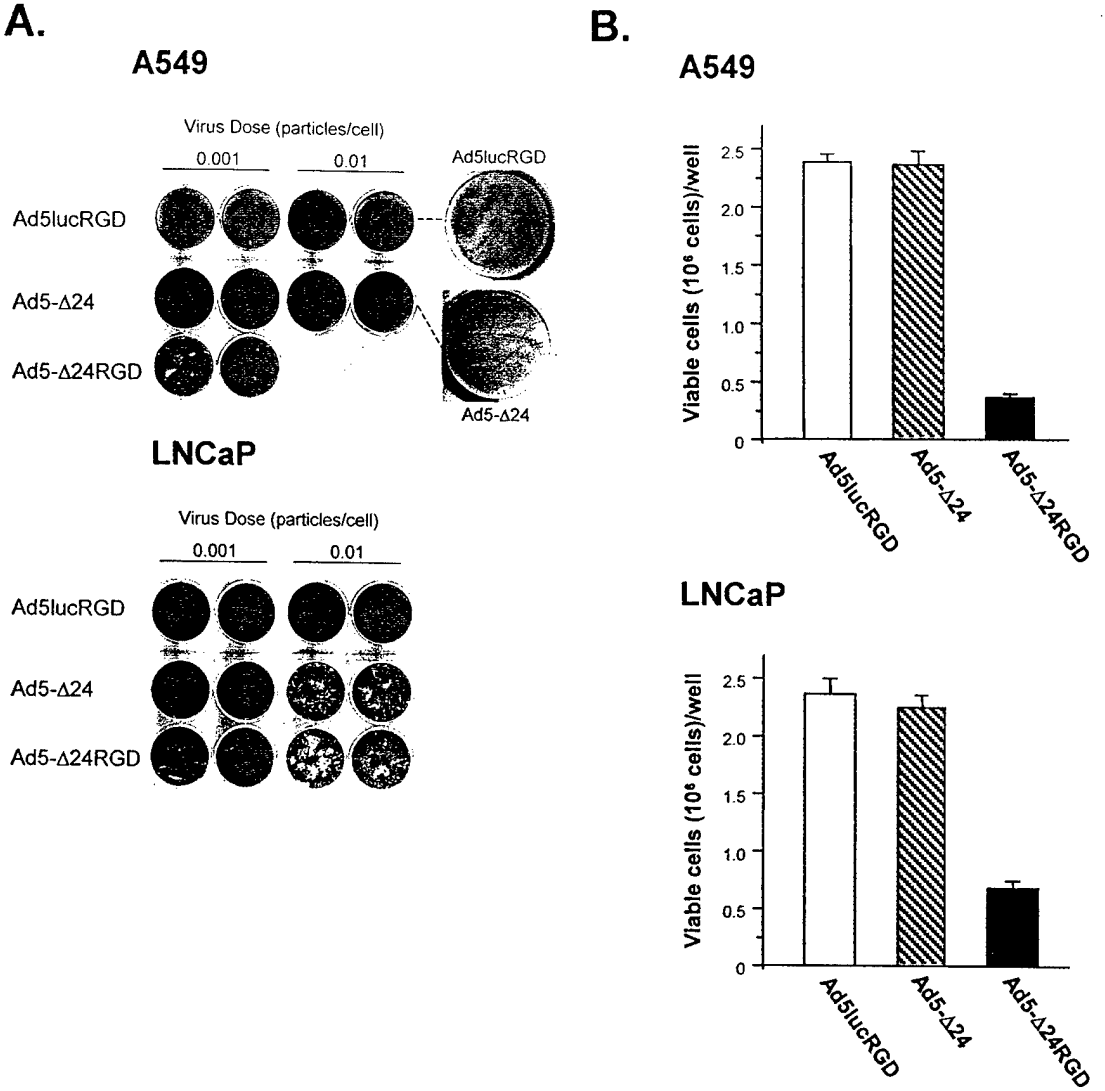

Fig. 3 Oncolytic potency of the RGD-modified virus. In *A*, A549 and LNCaP cells were infected with 0.001 or 0.01 particles/cell Ad5lucRGD, Ad5-Δ24, or Ad5-Δ24RGD. Eight (*A549*) and 10 days (*LNCaP*) later, the cells were fixed and stained with crystal violet. A higher magnification of two wells is presented to show the incipient cytopathic effect of Ad5-Δ24. In *B*, in parallel, cell viability was analyzed with an XTT colorimetric assay. In both cell lines, Ad5-Δ24RGD had higher lytic potency than did its unmodified counterpart, as shown by the percentage of viable cells remaining in the corresponding treatment conditions.

Ad5lucRGD, Ad5-Δ24, or Ad5-Δ24RGD in A549 cells at 8 days after infection by plaque assay. Ad5-Δ24RGD produced a viral yield of 3.75×10^9 plaque-forming units/ml which was 43 times higher than that of its unmodified Ad5-Δ24 counterpart (8.75×10^7 plaque-forming units/ml). No virus was obtained from the nonreplicative control Ad5lucRGD-infected cells. These results are consistent with the fact that modifying the fiber knob with an RGD motif led to enhancement of viral infectivity and an increase in the production of infectious adenovirus.

Increased Oncolytic Potency of Infectivity-enhanced CRAd *in Vitro*. To demonstrate the increased lytic potency of Ad5-Δ24RGD, we infected A549 and LNCaP cells with small amounts of each virus to allow multiple cycles of viral replication over the ensuing 8 days, then stained the attached cells with crystal violet and counted viable cells by XTT assay. In both cell lines, the fewest viable cells were detected in the Ad5-Δ24RGD-infected group (Fig. 3, *A* and *B*). The cell lysis capacity of Ad5-Δ24RGD is 7 times higher in A549, and 3.5 times higher in LNCaP compared with Ad5-Δ24. These results demonstrate that the fiber knob modification enhanced adenoviral lytic potency over that of the Ad5-Δ24 virus.

Increased Oncolytic Potency of Infectivity-enhanced CRAd *in Vivo*. The ultimate goal of this study was to demonstrate the oncolytic superiority of infectivity-enhanced CRAds over that of unmodified adenoviruses *in vivo*. Because low doses of virus allow several cycles of replication along with destruction of tumor cells, even a single dose

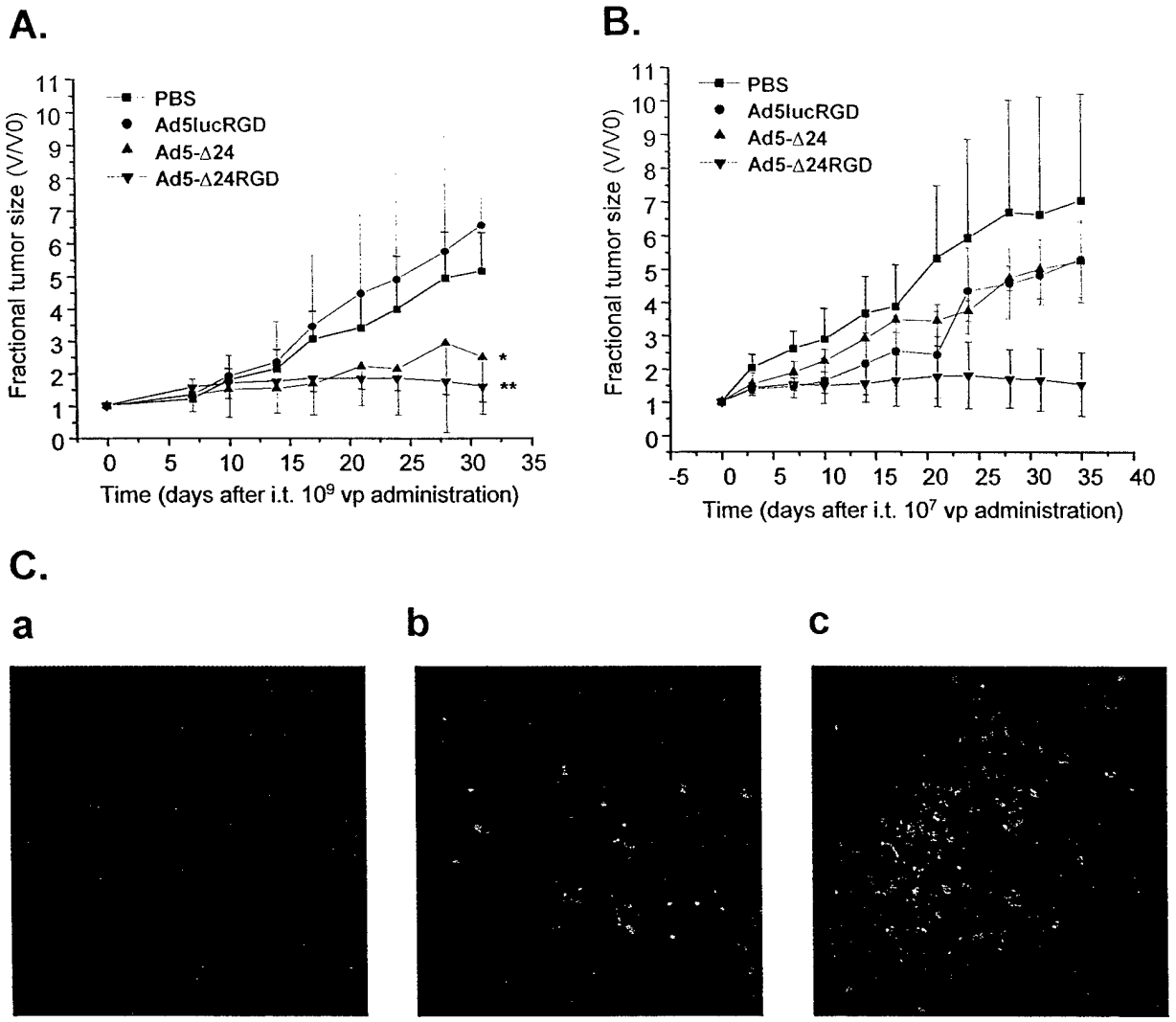

Fig. 4 *In vivo* oncolysis by high and low doses of infectivity-enhanced CRAds. s.c. A549 xenografts in nude mice were treated with a single i.t. injection of (*A*) 10^9 viral particles or (*B*) 10^7 viral particles of Ad5lucRGD, Ad5-Δ24, or Ad5-Δ24RGD, or with PBS alone. Tumor size was measured twice a week. Results are shown as fractional tumor volumes (*V/V0*, where *V* = volume at each time point, and *V0* = volume at adenovirus injection); *each line*, the mean of five tumors (±SD) in the high-dose group, and 4 tumors (±SD) in the low-dose group. In the high-dose experiment, both CRAds show a similar oncolytic effect that results in smaller tumors compared with PBS-treated groups (*, Ad5-Δ24, $P < 0.05$; **, Ad5-Δ24RGD, $P < 0.01$). However, in the low-dose experiment, tumors treated with Ad5-Δ24 followed a growth curve similar to that of tumors treated with nonreplicative Ad5lucRGD, whereas tumors treated with Ad5-Δ24RGD did not grow ($P < 0.01$ compared with PBS). *C*, detection of adenovirus hexon in tumor xenografts by immunofluorescence. Frozen sections of tumor specimens injected with (*a*) Ad5lucRGD, (*b*) Ad5-Δ24, and (*c*) Ad5-Δ24RGD were treated with goat antihexon antibody and Alexa Fluor 488-labeled donkey antigoat antibody, and nuclei were counterstained with Hoechst 33342. Images were captured from Leitz fluorescence microscope (×100) with a double filter. Sections taken from tumors treated with CRAds were positive for adenovirus presence (*green dots in b* and *c*); Ad5-Δ24RGD signal was stronger than that of Ad5-Δ24. Samples taken from tumors treated with PBS (not shown) or Ad5lucRGD exhibited no hexon signal (*a*). *vp*, viral particles; *Ad*, adenovirus.

would produce an exponential rise in the number of killed cells, which would extend to the entire tumor. To demonstrate this hypothesis, we treated A549 xenografts in nude mice with a single i.t. injection (10^9 viral particles) of one of the three viruses or with PBS. At 32 days after injection, both CRAds had an oncolytic effect in the tumors opposite to that of those treated with nonreplicative virus or with PBS (Ad5-Δ24, $P < 0.05$; Ad5-Δ24RGD, $P < 0.01$ compared with PBS group; Fig. 4*A*). Given these results, we conducted another experiment in which we administered a 100-fold lower dose (10^7 viral particles) of the viruses. At this dose, Ad5-Δ24 treatment did not show a statistically significant difference compared with either PBS or AdlucRGD. However, it demonstrated that the oncolytic effect of Ad5-Δ24RGD is maintained (Ad5-Δ24RGD *versus* PBS, $P < 0.01$; Ad5-Δ24RGD *versus* Ad5-Δ24, $P < 0.05$). These variations observed between high-dose and low-dose experiments suggest that a threshold dose over 10^7 viral particles of Ad5-Δ24 is required

to obtain an oncolytic effect in tumor nodules (Fig. 4*B*). To confirm that the CRAds were present in the tumor tissue, we used immunofluorescence to detect the virus hexon in tumor samples collected after the low-dose experiment (35 days postinjection). Ad5-Δ24RGD was present in the tumor nodules, as was Ad5-Δ24 to a lesser extent. PBS- and Ad5lucRGD-treated nodules showed no hexon signal (Fig. 4*C*). These results corroborated the observation that the partial reduction of tumor mass was attributable to virus replication and that the RGD modification of the fiber knob conferred infectivity and oncolysis advantage to a CRAd *in vivo*.

DISCUSSION

CRAds are novel and promising agents for cancer therapy. However, their efficacy is predicated on efficient tumor infection, specific replication, and lateral spread. The deficiency of CAR in a variety of tumor targets is a limitation to adenovirus infection. In a previous report, we demonstrated that the insertion of an RGD motif into the HI loop of the fiber knob of nonreplicative adenoviruses enhances tumor infection (16, 17). This proves that CAR-independent entry represents a viable way to circumvent CAR deficiency in some tumor types.

In this report, we have demonstrated that the genetic introduction of an RGD sequence in the fiber of a CRAd, such as previously characterized Ad5-Δ24 (18), allows CAR-independent infection that leads to the enhancement of viral propagation and oncolytic effect *in vitro* and *in vivo*. The increased initial virus entry into the cells rendered by the RGD modification results in earlier detection and augmented yields of encapsidated DNA of Ad5-Δ24RGD compared with the unmodified Ad5-Δ24 (Fig. 2). Because this tropism modification is not anticipated to alter fundamental aspects of the viral replication cycle, this effect was likely attributable to the infectivity enhancement allowed by delivering the virus through CAR-independent pathways. Subsequently, we studied the oncolytic potency of CRAds in two cell lines and concluded that Ad5-Δ24RGD potency is higher than that of the unmodified virus. Although the XTT assay was not sensitive enough to demonstrate the lytic effect of Ad5-Δ24 compared with the nonreplicative Ad5lucRGD, the crystal violet showed early comet-like cytopathic areas in Ad5-Δ24-treated A549 and LNCaP cells, which indicated the presence of an incipient lytic effect, whereas Ad5lucRGD-treated cells were intact (Fig. 3*A*). The less notable difference between Ad5-Δ24RGD and Ad5-Δ24 seen in LNCaP cells is explained by the absence of the $\alpha_v\beta_3$ integrins (25), compensated by the presence of other types of RGD-binding integrins ($\alpha_3\beta_1$ and $\alpha_5\beta_1$; Ref. 26) that were rapidly saturated (Fig. 3).

Our ultimate goal was to demonstrate the superior oncolytic effect of Ad5-Δ24RGD in an *in vivo* model. To this end, A549 cells xenografted in nude mice were treated with single, high-dose (10^9 viral particles), i.t. injections of Ad5lucRGD, Ad5-Δ24, Ad5-Δ24RGD, or PBS, and the results showed that both CRAds (modified and unmodified) yielded similar oncolysis (Fig. 4*A*). However, when a 100-fold lower dose (10^7 viral particles) was administered, it became clear that the oncolytic effect of Ad5-Δ24RGD was higher than that of Ad5-Δ24 ($P < 0.05$; Fig. 4*B*). Furthermore, we were able to correlate the observed oncolytic effect with the presence of virus progeny in the tumor samples by immunofluorescent detection of adenoviral hexon. Hexon was not detected in PBS- (not shown) and Ad5lucRGD-treated nodules (Fig. 4*C*, *a*), whereas it was detected throughout the tumors treated with CRAds. The comparison between the two CRAds showed that fluorescence in Ad5-Δ24RGD-treated tumors was stronger than the one observed in Ad5-Δ24-treated tumors (Fig. 4*C*, *b* and *c*, respectively). The lack of fluorescent staining in tumors treated with the nonreplicative control Ad5lucRGD indicates that the detected hexon belongs to the viral progeny of Ad5-Δ24 and Ad5-Δ24RGD, and not to the initial inoculum. As regards the high divergence of the volumes of PBS- and Ad5lucRGD-treated tumors, factors such as highly heterogeneous cell replication rates and hypoxic and necrotic areas are known to affect individual tumor volume after a critical size is reached. These differences have been noted previously when using oncolytic viruses (27, 28). Nevertheless, total resolution of the tumors in the s.c. xenograft model was seen only in some nodules treated with Ad5-Δ24RGD, which indicated that administration volume and schema adjustments, such as the ones suggested recently by Heise *et al.* (29), might be necessary to achieve complete oncolysis.

As presented here and elsewhere (30), the efficacy of replication-competent viruses used as oncolytic agents can improved at the level of infectivity. As other tumor-binding peptides are isolated (30, 31), modifications in addition to the RGD insertion can be considered as well. Of note, the RGD modification described here does not preclude the binding of the fiber to CAR, and the modified virus can enter the cells through α_v integrins and CAR. One approach to improve specific tumor infection/transduction would be the combination of CAR ablation and tumor-specific ligands to redirect the virus tropism. Recently, the adenovirus fiber amino acids crucial for CAR-binding abrogation and new tumor-selective peptides have been defined (15, 31, 32). This combination will generate truly targeted viruses, but the efficiency of their propagation will depend on the amount of the targeted receptor in the same way as the propagation of the unmodified virus depends on CAR.

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Minireview

The Development of Conditionally Replicative Adenoviruses for Cancer Therapy[1]

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Abstract

Replicative viral agents represent a novel approach for treating neoplastic disease. Tumor cell killing by the viral agent is achieved by direct consequence of the viral replication. Relative sparing of nontumor is, however, required to provide a therapeutic index of utility for cancer treatment. To this end, an ideal viral agent would, thus, possess several logical attributes, including stability and efficiency for infection and lateral spread *in vivo*, a preference for replication in tumor *versus* nontumor cells, and the capability of avoiding early detection—and eradication—by the immune system. To date, none of the agents has exhibited optimal characteristics with regard to the aforementioned attributes. Adenovirus, however, has lent itself to a process of extensive engineering that is dealing with each and every one of the major requirements and that is realizing its clinical potential. An advanced understanding of the cancer phenotype, as well as achievements in functionally exploiting viral plasticity, predicate the design and realization of conditionally replicative adenoviral agents with improved characteristics for cancer therapy.

Introduction

The use of replicative viral agents represents a novel approach to neoplastic disease. In this strategy, target tumor cell killing by the viral agent is achieved by direct consequence of the viral replication (1). Furthermore, relative sparing of nontumor cells provides a therapeutic index of potential utility for cancer treatment. On this basis, it is apparent that the specificity of the viral agent for achieving tumor cell killing via replication ("oncolysis") is the functional key to successful exploitation of these agents for therapy. To this end, an ideal viral agent would, thus, possess several logical attributes: (*a*) such viruses must have the capacity to infect target cells *in situ*, that is, within the stringency imposed by direct *in vivo* delivery. Thus, a level of stability in the *in vivo* context is mandated to achieve an effective initial inoculum. Furthermore, such stability in the *in vivo* context would be critical for allowing replicated viruses to infect laterally, a key process to realizing effective amplification; and (*b*) the viral agent should possess a relative preference for replication in tumor *versus* nontumor cells. Thus, a useful viral agent would be well characterized in terms of entry biology and replicative physiology, such that these steps might be modified to achieve the desired tumor cell specificity, if thus required. Specifically, modulation of viral tropism, either by alteration of the initial attachment/entry steps or by modification of the functional aspects of viral genome replication and progeny-virus packaging, offers a means to achieve such specificity. Another potentially useful property for replicative viruses would be the capability of avoiding early detection and eradication by the immune system. Although a variety of viral agents have been used as replicative agents—including Bunyamwara, Coxsackievirus, dengue, mumps, Newcastle disease virus, vaccinia, West Nile virus, and adenovirus—none of the agents has exhibited optimal characteristics *vis-à-vis* the aforementioned desired attributes (2–4).

Attributes of Adenovirus Recommend Its Use

With respect to candidate replicative viral agents, adenoviruses possess many relevant attributes that recommend their use in this context (5). In this regard, adenoviral vectors have been used extensively for a variety of gene therapy applications (6, 7). In these various gene therapy schemas, adenovirus has exhibited an unparalleled efficiency allowing effective infection of target cells in the context of *in vivo* gene delivery. This attribute would logically predicate the ability of replicative adenoviruses to achieve a high initial inoculum to target tumor cells when used as a replicative agent. Of note, the entry pathway of the virus has been extensively characterized (8). On this basis, tropism modifications of the adenovirus have allowed rerouting of the virus through heterologous cellular pathways to allow achievement of cell specific gene delivery (9). Such biological plasticity would thus, in theory, allow infectious specificity to be achieved via restriction of binding exclusively to tumor cells. In addition, the replication cycle of the adenovirus has been the subject of investigation for several decades (10). Consequently, there exists a large database of information with respect to the viral regulatory mechanisms involved in the replicative cycle (11, 12). Thus, from the standpoint of inoculum efficiency and replicative specificity, adenovirus vectors offer potential utility as a conditionally replicative viral agent by providing the basis by which to modify the parent virus toward the requirements of a true CRAD reagent.

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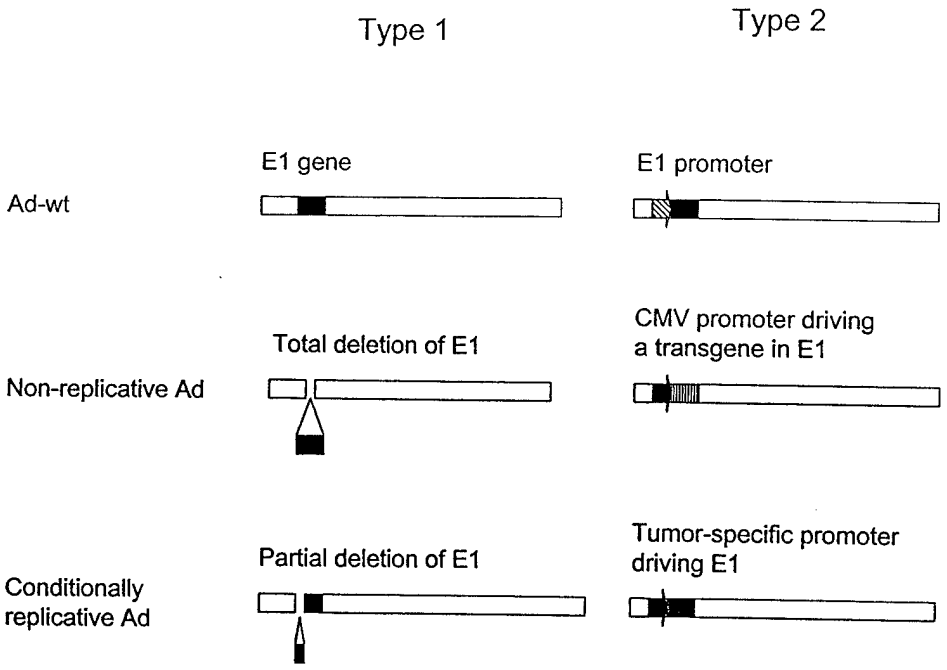

Fig. 1 Schematic representation of types of CRAD agents. Depicted are genomes of CRAD agents with illustration of the basis of conditional replication. For Type I CRADs, the design strategy of the transcomplementing genome is shown. Wild-type adenovirus (*Ad-wt*) has an intact *E1* gene that triggers early gene expression and adenovirus replication. A nonreplicative adenovirus (Ad) has a complete deletion of *E1A* and thus cannot propagate except in the context of *E1A*-expressing packaging cells. A conditionally replicative Ad may be derived by partial deletions of *E1* in which tumor cells provide the missing functions to allow replication. Ideally, the transcomplementing functions will be present in tumor cells but not in nontumor cells. For Type II CRADs, conditional expression of the *E1A* gene is achieved via a tumor-specific promoter. To achieve this end, replacement of the native E1 promoter with the tsp would ideally allow *E1* expression only in promoter-inductive tumor cells. This *E1* expression could then trigger a replicative cycle for the adenovirus. Again, tumor-selective induction of the promoter is the basis of specificity. CMV, cytomegalovirus.

Engineering Conditionality of Replication

Specificity of Replication Based on Tumor Biology. Initial attempts to derive CRADs[3] focused on the achievement of tumor selective replication (6). In this regard, by using the knowledge that components of the adenovirus replication cycle intrinsically interact with specific functional cellular proteins, one strategy has been the generation of CRAD vectors targeted to biological factors modified in cancer cells (Fig. 1). One such attenuated virus, containing mutations within an adenoviral early-transcribed gene, was developed to replicate only in cells lacking the cell cycle control protein p53 (6). Of note, cell cycle regulatory proteins, such as p53, are mutated in nearly all actively growing tumors (13); thus, the dependence of viral replication on the presence or absence of these proteins represents an ideal regulatory mechanism that potentially provides tumor-specific replication. On this basis, a mutated adenovirus, termed dl1520, was derived that contains two deletions within the *E1B-55* gene. Initial studies carried out with this agent demonstrated therapeutic potential, with the achievement of tumor regression and even complete elimination of tumors in some murine xenograft models (6, 14). These findings resulted in the rapid translation of the virus into human Phase I, and then Phase II, clinical trials for carcinoma of the ovary and of head and neck cancer treatment (15). Of note, however, studies by Turnell *et al.* (16), and Goodrum *et al.* (17) determined that actual specificity of viral replication of dl1520 is not attributable to the absence or presence of p53 but is based on the timing of viral replication in tumor cells or other undefined (18, 19) factors. Replication of dl1520 is, therefore, not strictly linked to the presence of p53. In addition, replication in normal human primary cells has been noted (20). Thus, though the initial concept of targeting replication to the presence of a functional *p53* gene was not realized with this virus, empiric efficacy in tumor treatment has been suggested.

Specificity of Replication Based on Transcriptional Control. Given the inability to achieve absolute specificity with engineered replicative viruses via the aforementioned approach, investigators have used other methods (Table 1). In this regard, an alternate means for obtaining tumor specific adenoviral replication has been developed based on exploiting heterologous transcriptional control regions, or promoters, to restrict replication of the adenovirus to tumor. This has been accomplished by placing an essential adenoviral gene under the control of a heterologous genetic regulatory element the expression of which is limited to specific tissues or tumors. Two groups have

[3] The abbreviations used are: CRAD, conditionally replicative adenovirus/adenoviral; PSA, prostate-specific antigen; HCC, hepatocellular carcinoma; CAR, Coxsackie and adenovirus receptor.

Table 1 CRAD agents

Agent name[a]	Regulation method	Adenoviral region	Cancer type	Anti-tumor action	Research (Ref.)	Studies
CN706	TRAG[b]	E1A	Prostate	Oncolysis	Rodriguez *et al.* (7)	Human trials
787	TRAG	E1A and E1B	Prostate	Oncolysis	Yu *et al.* (24)	Animal models
AvE1a04I	TRAG	E1A	Hepatocellular carcinoma	Oncolysis	Hallenbeck *et al.* (21)	Animal models
Adv-E1AdB-F/K20	AGDCC	E1A	Glioblastoma	Oncolysis	Shinoura *et al.* (44)	Cell culture
Ad.TK^{RC}	AGDCC	E1B	Colon	Oncolysis and toxin (thymidine kinase)	Wildner *et al.* (28)	Animal models
DI1520	AGDCC	E1B	Head and neck Ovary	Oncolysis	Bischoff *et al.* (6)	Human trials
Onyx-15, CD/HSV-1, TK	AGDCC	E1B	Cancer cell lines	Oncolysis and toxin (thymidine kinase and cytosine deaminase)	Freytag *et al.* (27)	Cell culture
Ad5dI309	AGDCC	E1 and E2	Cancer cell lines	Oncolysis	Medina *et al.* (45)	Cell culture

[a] Conditionally replicative adenoviruses engineered to date.
[b] TRAG, transcriptional regulation of adenovirus genome; AGDCC, adenoviral genome deleted to complement cellular genotype.

demonstrated the validity of this model by using such tumor-specific transcriptional regulatory elements, which control the essential early adenoviral genes (Table 1; Refs. 7, 21). In these instances, practical considerations dictated the strategy of heterologous control of the *E1A* gene. In addition, direct antitumor affects of E1A, based on apoptosis induction may be exploited in this manner (22). In this regard, the existence of E1A-transcomplementing cell lines, plus available plasmid packaging systems (23), allows for facile construction and rescue of such recombinant adenoviruses.

A variety of CRAD strategies have exploited this design strategy. In this regard, recognizing that levels of PSA are elevated in the prostate of individuals with prostate cancer, the transcriptional promoter sequences of the PSA gene have been configured into adenoviral vectors to regulate E1 transcription (7). In mouse xenograft models, this replicative adenovirus eradicated large PSA-expressing tumors and abolished PSA production with a single intratumoral injection. Yu *et al.* (24) have presented studies using a CRAD vector containing dual promoter regulation within the E1 region with promoters separately controlling expression of E1A and E1B. This replicative adenovirus was demonstrated to lyse PSA expressing cells with a selectivity of 10,000-fold over that of non-PSA-expressing cells. An alternative approach uses sequences that drive the expression of the HCC marker *α-fetoprotein*, a gene that is singularly expressed in dividing hepatocytes and HCC (21). In addition, binary systems have also been developed as a means to achieve delivery that transcomplements E1A (25, 26).

Multimodality Treatments

In addition to use as single agents, replication-competent adenoviruses have also been exploited in the context of combination treatment with conventional anticancer approaches. In this regard, several groups have examined the efficacy of this approach by configuring a toxin gene, such as *cytosine deaminase* or herpes *thymidine kinase*, into the context of replicative adenoviruses. In addition, Freytag *et al.* (27) have developed a replicative adenovirus that is configured with a *thymidine kinase/cytosine deaminase* fusion gene . The resultant toxin product kills cells with the administration of the prodrug, besides increasing the sensitivity of the tumor to radiation. Wildner *et al.* (28, 29) and Heise *et al.* (30) have demonstrated that both of the therapy schemes bring additive effects to replicative viral cancer therapy. Furthermore, the resultant bystander effect seen from toxin-expressing cells is such that nontransduced tumor cells may likewise be eradicated, thereby accomplishing an additional mechanism for the achievement of an amplified antitumor effect. It has been proposed that utilization of this method may add a measure of safety to the use of oncolytic viruses in that one can effectively control the spread of virus via the addition of the prodrug analogue, which would selectively ablate virus-infected cells.

Obstacles for Clinical Application of CRADs

Despite the various theoretical advantages of replicative adenoviral agents, the various strategies for use of CRADs will only allow true utility if they account for all of the relevant aspects of tumor biology.

Scarcity of Adenoviral Receptors in Human Tumors. From the standpoint of inoculum efficiency, it has been noted that primary tumor is relatively refractory to adenoviral infection compared with cell line counterparts. This phenomenon is shown to occur on the basis of a relative deficiency of the primary adenovirus receptor CAR (31, 32). Clearly, the resistance of tumor targets to adenoviral infection will restrict not only the efficiency of the initial inoculum but also the ability of the virus to infect laterally postreplication. On this basis, in the absence of CRAD vectors that will infect with true tumor cell specificity, replicative adenoviral agents will at least need to possess the ability to achieve CAR-independent gene transfer (31). Indeed, such fundamental limits as tumor refractoriness to adenoviral infection may represent the major barrier to realizing the full benefit of CRAD agents translated into the clinical context at this point.

True Tumor Specificity. From the standpoint of replicative specificity, a number of design aspects used to date potentially undermine the goal of true tumor specificity. In the first regard, although transcomplementation of E1A offers practical advantages, a number of limits must be taken into account. In this regard, a number of tumors exhibit E1A-like activity and are, thus, capable of transcomplementing E1A(−) viruses (33, 34). Indeed, this capacity has actually been exploited in the

design of a class of CRAD agents that exploit interleukin 6-inducible E1A-like activity (33). The presence of intrinsic E1A-like activity would clearly operate to undermine the design of CRAD agents with E1A under control of tumor-specific promoters. In addition, promoter function in the adenoviral genome context is idiosyncratic, as has been noted in the context of a variety of adenoviral vectors designed to achieve transcriptional targeting of transgenes to tumor cells. Furthermore, this dysregulation of promoters is likely to be of even greater consequence in the context of cellular physiology induced by the replicative cycle of adenovirus. To address this, specific endeavors to understand heterologous promoter function in a CRAD context must be undertaken. Additional steps to maintain the fidelity of such promoters will require development and validation. Although some initiatives in this direction have been applied for adenovirus vectors, their relevance for CRAD vectors remains to be determined.

Adenoviral Interaction with the Immune System. Another key factor relevant to realizing the full therapeutic potential of CRAD agents is the interaction of the adenovirus with the immune system. In this regard, therapeutic efficacy of replicative adenovirus is predicated on the idea that replication and lateralization within tumors could occur without impairment via host eradication of the virus by immune mechanisms. Of note, Bramson *et al.* (35) have suggested that the intratumoral environment is a relatively privileged site in regard to adenoviral interaction with the immune system. Thus, appropriate physiology may exist within the tumor to allow further gain in viral amplification. On the other hand, Ikeda *et al.* (36). have shown that immunosuppression limits the utility of replicative herpes virus for antitumor therapy. On this basis, it may be argued that steps to attenuate the host immune response to adenovirus are rational. Although a variety of immunological approaches have been used to try to limit the host immune response to adenoviral vectors (37, 38), their use in the context of replicative adenoviruses raises particular safety concerns. Furthermore, at this time, mouse and rat tumors do not support efficient replication of human adenoviruses, so that syngeneic immunocompetent rodent tumor models are not available to evaluate the interaction between CRAD and the human immune system. Clearly, future studies are necessary to address the issue of immunomodulation of CRADs.

The Clinical Indications for Using CRADs

Clinical translation of CRAD agents has progressed rapidly through Phase I and Phase II trials. These efforts have largely been carried out in the context of local or locoregional disease. This fact reflects the verity that the current generation of CRAD agents generally exhibits the promiscuous tropism of parent adenoviruses. On this basis, tumor-specific delivery is restricted to anatomical locations whereby the virus may be delivered and contained locally. This aspect of CRADs has limited the use of these agents for disseminated diseases, in which systemic delivery would be mandated. Thus, the ability to achieve cell-specific gene delivery via tropism modification of the parent virus would be required to allow the application of CRAD agents in the important context of disseminated disease (39). One key aspect of such a scenario is that the amplifying principle nature of CRADs may allow the use of a much lower dose of administered adenovirus. On this basis, it may, in fact, be more feasible to use CRADs in a systemic manner for disseminated disease than to use adenoviral vector counterparts. This is especially relevant in the context of severe host reaction to i.v. injected adenovirus limiting the therapeutic efficacy of treatment (40).

Conclusion

Despite these caveats, CRADs clearly represent antitumor agents of exciting promise. A greater understanding of precise patterns of tumor-specific gene expression will clearly offer additional venues for the derivation of viral tumor-specific replication. These endeavors will likewise be fostered by technologies to improve promoter specificity—via direct engineering of the adenoviral genome (41, 42) as well as via shuffling—and promoter evolution methods (43). In addition, dramatic strides have been made in adapting adenoviral vectors for cell-specific gene delivery. Clearly, these technologies will complement recent National Cancer Institute-directed efforts to a full characterization of unique surface molecules that distinguish tumor cells. Thus, on this basis, an advanced understanding of the cancer phenotype, as well as achievements in functionally exploiting viral plasticity, predicates the design and realization of CRAD agents with more improved characteristics for cancer therapy.

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ORIGINAL

Strategies to Adapt Adenoviral Vectors for Targeted Delivery

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ABSTRACT: **The utility of current generation adenoviral vectors for targeted, cell-specific gene delivery is limited by the promiscuous tropism of the parent virus. To address this issue, we have developed both genetic and immunologic methods to alter viral tropism. Immunologic retargeting has been achieved via conjugates comprised of an antifiber knob Fab and a targeting moiety consisting of a ligand or antireceptor antibody. Gene delivery by this approach has been accomplished via a variety of cellular pathways including receptors for folate, FGF, and EGF. In addition to cell-specific gene delivery, this strategy has allowed enhanced gene delivery to target cells lacking the native adenoviral receptor, CAR. Of note, this specific and extended gene delivery allowed enhanced survival in murine models of human carcinoma via cancer gene therapy. Genetic strategies to alter adenoviral tropism have included both fiber modification and fiber replacement. In the former, we have identified the HI loop of fiber as a propitious locale for introduction of heterologous peptides. Incorporation of an RGDC peptide at this locale allowed gene delivery via cellular integrins with dramatic efficiency augmentations. As a strategy to achieve both new tropism as well as to ablate native tropism, methods have been developed to replace the fiber protein with heterologous motif which preserves the key trimeric quaternary structure of fiber and allows for propagation. Such a fiber-replacement virus has been rescued and has demonstrated capacities consistent with its utility as a novel vector agent. These strategies have allowed the achievement of cell-specific gene delivery via adenoviral vectors and thus have the potential to enhance the utility of this vector agent.**

INTRODUCTION

For the effective application of gene therapy strategies to human disease, Anderson[1] suggested certain criteria should be met, namely, that vectors should deliver a therapeutic gene specifically to a target cell, that resultant gene expression should be at an appropriate level and for an appropriate period of time, and that delivery and expression of the therapeutic gene should be achieved within an acceptable safety margin.[1] These criteria remain largely unmet. However, in recent years disappointment in the results of clinical trials has forced a refocus on the basics of vector design, resulting in steady advancements in vector technology, which now show promise for more successful gene therapy.

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Development of vectors that have *in vivo* efficacy is critical, because many diseases for which gene therapy can rationally be considered require direct *in situ* gene delivery and cannot feasibly be addressed by an *ex vivo* approach. Replication incompetent adenovirus is a potential candidate vector for clinical gene therapy based on several key attributes, including ease of production to high titer, infection of both dividing and nondividing cells, and systemic stability, which has allowed for efficient *in vivo* gene expression.[2] However, the virus has several important limitations including its widespread tropism, stimulation of inflammatory and immune responses, and short-term transgene expression.[3–5] This article focuses chiefly on the issue of targeted gene delivery to address the limitations brought about by native viral tropism. To date, several groups have sought to exploit the fundamental advantages of adenovirus by using it in specific contexts where the recognized limitations were judged to be less important. For example, it was thought that the issue of the widespread tropism of the virus could be circumvented by administering the vector by direct injection, particularly in the context of tumors. However, in phase I human trials, dissemination beyond the injected site was found. Application to "compartmentalized" disease has also met with problems. For example, poor gene transfer efficiency has been noted following administration into the pleural space for therapy of mesothelioma[6] (S.M. Albelda, unpublished data), and in the peritoneum, effective use of antitumor gene therapy has been limited by concurrent gene transfer of the liver with subsequent toxicity.[7] Further limitations have arisen in the application to pulmonary disease. Here, prior clinical experience had indicated that the virus had a natural tropism for the respiratory tract; therefore, direct administration of vector to the airways for cystic fibrosis therapy seemed a rational approach.[8–12] In reality, the achieved levels of gene transfer were lower than expected, because differentiated airway epithelial cells lack sufficient adenoviral receptors and the integrins required for viral internalization.[13–16] Therefore, even in these apparently favorable anatomic locations there is a strong case for developing a vector with cell-specific targeting properties. Despite the limitations of adenovirus, its basic advantages, particularly its *in vivo* efficacy, justify using this virus as a starting point in the development of improved vector systems.

ADENOVIRAL ENTRY PATHWAY

Strategies for retargeting viral vectors were first applied to retroviruses and were based on a sound understanding of viral entry mechanisms.[17] The entry mechanisms of adenovirus, including the recent identification of primary adenoviral receptors, are now well understood and allow for a rational approach to the targeting of adenoviral vectors (FIG. 1).

The adenovirus is an unenveloped icosahedral particle with 12 fibers projecting from the surface.[18] During the assembly phase of viral replication, fiber monomers trimerize in the cytoplasm, then bind to a viral penton base protein that is subsequently incorporated into the viral capsid. At the distal tip of each fiber monomer is a globular region referred to as the knob domain. It is this knob region that binds to cellular adenoviral receptors, initially anchoring the virus to the cells. Two cellular receptors for adenovirus were recently described. The coxsackie/adenoviral receptor

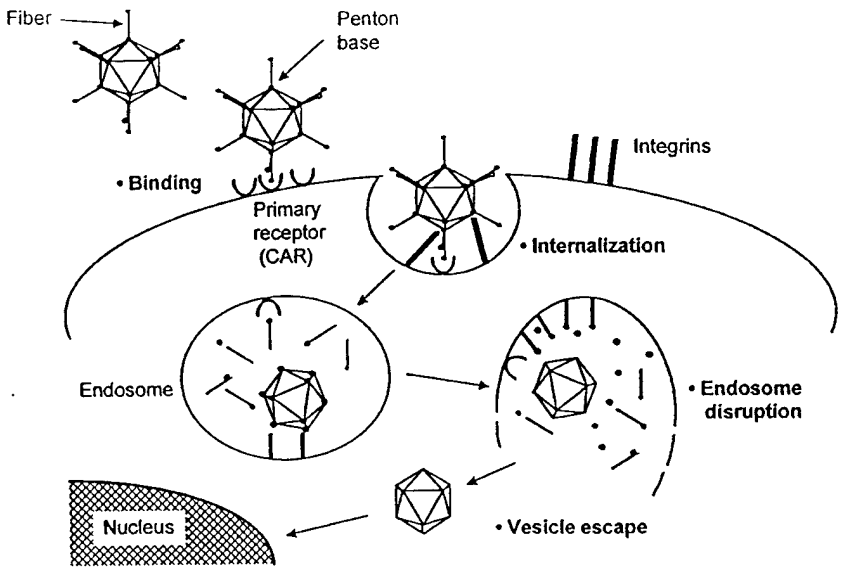

FIGURE 1. Adenovirus infection: binding and entry pathway.

(CAR)[3,19] binds both adenovirus and group B coxsackie viruses. The murine and human receptors are 365 and 352 amino acids, respectively, and 91% identical. The extracellular region appears to contain two immunoglobulin-like domains. In a separate report, viral binding to the α2 domain of major histocompatability complex class 1 was also shown.[20] Following attachment, viral entry requires a second step, which involves the interaction between Arg-Gly-Asp (RGD) motifs in the penton base with cell surface integrins αvβ3 or αvβ5, which then leads to receptor-mediated endocytosis of the virion.[21] In the endosome the virus undergoes a stepwise disassembly, and endosomal lysis occurs (a process mediated by the penton base and low endosomal pH), followed by transport of the viral DNA to the cell nucleus. This endosomolysis step is critical for efficient gene delivery, and the ability of the adenovirus to effect endosomal escape is a key factor in its efficiency as a vector. Importantly, viral entry and endosomal escape are functionally uncoupled[22]; thus, entry via a non-native, cell-specific pathway does not appear to compromise downstream delivery of DNA to the nucleus. Based on the foregoing, a logical place to start in the development of a targeted adenoviral vector is manipulation of the knob domain. Several groups are now developing strategies to impart targeting ability to adenoviral vectors. The strategies currently being developed may be categorized as "immunologic" or "genetic."

IMMUNOLOGIC RETARGETING

Immunologic retargeting strategies are based on the use of bispecific conjugates, typically a conjugate between an antibody directed against a component of the virus

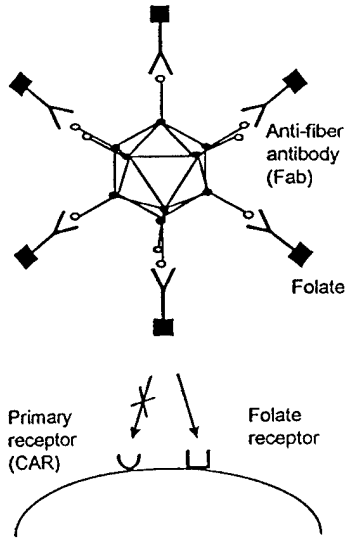

FIGURE 2. Schema for altering adenoviral tropism by an immunologic targeting approach. A retargeting moiety formed by conjugating folate to the Fab fragment of an anti-knob antibody is used to direct adenoviral binding to the folate receptor.

and a targeting antibody or ligand. True targeting requires simultaneous abolition of native targeting and introduction of new tropism; therefore, Douglas and colleagues[23] developed a neutralizing monoclonal antibody against the knob region of adenovirus. This was achieved by immunizing mice with adenovirus and recombinant knob protein, then developing hybridomas that produced antibodies capable of neutralizing native adenoviral infection (as determined by a cytopathic effect assay using HeLa cells). The Fab fragment of an antibody generated in this way (1D6.14) was then conjugated to folate to effect targeting to the folate receptor. Folate was chosen because the folate receptor is upregulated on several tumor types and the receptor internalizes after ligand binding.[24] Using this conjugate, adenoviral infection was redirected away from the native receptor to the folate receptor (FIG. 2). The gene transfer efficiency of this approach was approximately 70% of that seen with the native virus, which contrasted to the experience with retroviral retargeting where redirection had generally resulted in a dramatic fall in infectivity.[17] Although binding of the complex to cells was clearly mediated by folate, the mechanism of viral internalization was not established. In this regard, folate is normally internalized by potocytosis and enters the cell via a caveolus.[25] Normally the size of this caveolus would be too small to encompass the folate-virus complex, but whether it could enlarge under these circumstances or whether viral entry was effected by the usual integrin-mediated pathway is unknown. Using a slightly different approach, Wickham and colleagues[26] developed an adenovirus that contained a FLAG domain introduced into the penton base region and then used a bispecific antibody directed against FLAG and αv integrins to direct binding to integrins on endothelial and

smooth muscle cells. Because the retargeting bispecific conjugate was larger than the viral fiber, it was hypothesized that the conjugate would be functionally available for binding by extending outward past the knob domain. In this way, by using integrins as the attachment target on these cells that express low levels of CAR, gene delivery was enhanced.

Further development in the immunologic retargeting approach was reported by Watkins and colleagues.[27] This group generated a bacteriophage library displaying single chain antibodies (sFvs) derived from the spleen of a mouse immunized against adenoviral knob. From this library a suitable neutralizing anti-knob sFv was isolated, and a fusion protein between this and epidermal growth factor (EGF) was then produced. This "adenobody" was successfully used to retarget adenoviral gene delivery, resulting in enhanced gene transfer of EGF receptor (EGFR)-expressing cells. Interestingly, this study showed that retargeting in this way appeared to bypass the need for the penton base/cell integrin interaction for internalization of the virus. This was shown by using an excess of an RGD-containing peptide that competes with penton for binding to integrins. This peptide reduced native viral gene transfer, but it had no effect on the gene transfer levels achieved with the retargeted vector complex, thus implying that in the latter case, viral entry was achieved by EGF receptor-mediated internalization. Taking a different approach to EGF receptor targeting, we used a conjugate between 1D6.14 Fab and a monoclonal antibody that binds to EGFR (mAb 425).[28,29] Using this approach, we demonstrated retargeted delivery to two murine fibroblast cell lines, one stably expressing human EGFR, the other expressing a mutant of EGFR that does not internalize (C.R. Miller, unpublished data). Therefore, depending on the target selected, it may be possible to exploit internalization mechanisms of either the targeted receptor itself or, if a noninternalizing target is selected, the native integrin pathway. The potential for overcoming a lack of integrins in some settings or the ability to target to noninternalizing cell surface molecules if integrins are sufficient implies a very broad potential applicability of retargeted vectors.

After the initial demonstration that immunologic retargeting of adenovirus could be achieved, further studies have explored the potential for therapeutic application of this approach. These applications might be considered in the context of several worthwhile goals of targeting with differing levels of stringency, including general nonspecific enhancement of delivery to a broad range of cell types, gene transfer of previously untransducible cells (relevant to both *ex vivo* and *in vivo* applications), targeting to enhance gene delivery and potentially reduce toxicity in a loco-regional or compartmental context, and cell-specific gene delivery following intravenous administration of vector.

At the lowest level of stringency, retargeting approaches to achieve enhanced gene delivery, even in the absence of a clear specificity advantage, are worthwhile in compartmentalized disease contexts. Such approaches at the very least should allow for the use of smaller doses of virus, thus potentially reducing direct viral toxic effects, dissemination from the administration site, and innate immune responses, which are clearly dose dependent. In this regard we used basic fibroblast growth factor (FGF2) as a targeting ligand, based on the knowledge that FGF receptors are upregulated in many tumor types. A conjugate between 1D6.14 Fab and FGF2 was used to retarget adenoviral infection of different tumor lines with varying baseline

levels of susceptibility to adenoviral infection. Enhancements in gene transfer from 2- to greater than 10-fold were seen (B.E. Rogers, unpublished data).

With regard to the gene transfer of resistant cells, Kaposi's sarcoma (KS) is an example of a disease in which gene therapy applications have been limited in part because of the poor transducibility of this tumor (J.A. Campain, unpublished data). Goldman and colleagues[30] used the FGF2 retargeting approach to investigate retargeted gene delivery to previously untransfectable KS cell lines. The results demonstrated a dramatic increase in gene transfer. Furthermore, the potential therapeutic utility was shown by transfecting cells with a retargeted adenovirus carrying the gene for herpes simplex thymidine kinase (AdCMVHSV-TK). These cells were then far more susceptible to killing by the subsequent administration of the pro-drug ganciclovir than were cells that had been infected with the untargeted virus. T lymphocyte is another cell type that is resistant to adenoviral infection because of the lack of both CAR and αv integrins. Thus, Wickham and colleagues[31] successfully retargeted adenoviral vectors by using a conjugate between the anti-FLAG antibody and anti-CD3, thereby achieving significantly enhanced gene transfer.[31] Thus, this study indicates that the benefits of targeting can also be exploited in contexts relevant to *ex vivo* therapy.

The ability to increase the number of genetically modified cells without resorting to an increase in viral dose is extremely important in view of the direct cytotoxic effects of adenovirus. For example, in the vasculature, when gene transfer is limited by a relatively low level of CAR expression,[26] gene transfer efficiency increases with escalating viral dose over a fairly narrow range, then it dramatically falls as cytotoxicity supervenes and leads to a loss of infected cells. For example, Schulick and colleagues[32] found maximal gene transfer efficiency in vascular smooth muscle cells of approximately 40% with 5×10^{10} pfu, which decreased to zero at 10^{11} pfu; similar results were found in the endothelium.[33] Using the bispecific anti-FLAG/anti-integrin approach, Wickham[26] achieved seven- to ninefold enhancement of endothelial cell gene transfer. As FGF receptor expression is upregulated in proliferating vasculature and is therefore relevant to several pathologic processes including tumor angiogenesis, we examined the effect of FGF2 targeting of adenoviral gene delivery to proliferating endothelial cells. Here, approximately 30-fold enhancement in luciferase expression was seen. Flow cytometry analysis of cells transfected with a β-galactosidase encoding vector demonstrated that FGF2 retargeting led to both an increase in the number of genetically modified cells and an increase in the amount of gene expression per cell. By contrast, when FGF2 retargeting was used in the infection of quiescent, confluent cells, transgene expression was actually reduced, indicating a degree of cell-specific targeting based on the level of expression of the targeted receptor (P.N. Reynolds, unpublished data).

Toxicity at high viral doses has also been seen in murine carcinoma models, in which escalating the dose of a herpes simplex thymidine kinase encoding virus (HSV-TK) eventually led to death from toxicity before complete tumor eradication could be achieved.[7] Also, in the context of HSV-TK, increasing the amount of transgene expression per cell (rather than the number of genetically modified cells) by manipulation of promoters did not increase the therapeutic effect once a threshold level of expression was achieved.[34] Thus, Rancourt and colleagues investigated the use of FGF2 retargeting of AdCMVHSV-TK in a murine model of ovarian carcino-

ma (C. Rancourt, unpublished data). First, FGF receptor expression on the ovarian carcinoma line SKOV3.ip1 was confirmed by radiolabeled FGF binding. Then, enhancement of adenovirally mediated gene delivery to these cells using FGF2 retargeting *in vitro* with the luciferase reporter gene was demonstrated. Next, validation of the retargeting approach *in vivo* was obtained. Tumors were established in nude mice by intraperitoneal inoculation with SKOV3.ip1 cells, followed in 5 days by a peritoneal injection of AdCMVLuc either alone or with FGF2 retargeting. Mice were sacrificed, and luciferase expression in the tumors was quantified. Tumors from mice that had received the retargeted vector had a 10-fold greater level of luciferase expression. Thus, these results importantly established that immunologic retargeting was efficacious *in vivo*. Tumors were then established in mice as before, followed by intraperitoneal injection of either placebo, AdCMVHSV-TK alone, or AdCMVHSV-TK with FGF2 retargeting (with viral doses of 10^8 and 10^9 pfu). Mice were then divided into two groups, received either ganciclovir or placebo for 14 days, and were monitored for survival. The mice that did not receive ganciclovir had no survival advantage over the mice that received no gene therapy. When survival curves for mice that received ganciclovir were analyzed, two important results emerged (FIG. 3). First, a statistically significant increase in survival occurred with

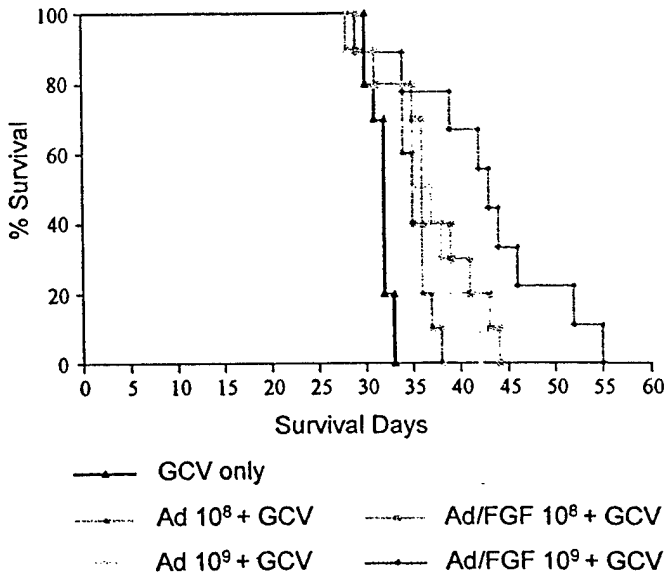

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FIGURE 3. Retargeting adenovirus to enhance therapeutic gene delivery enhances survival in a murine model of ovarian carcinoma. Mice with peritoneal ovarian tumors received intraperitoneal injections of placebo or adenovirus carrying the herpes simplex thymidine kinase gene (10^8 or 10^9 pfu), either unmodified virus (Ad) or virus complexed to a retargeting moiety (formed by conjugating FGF2 to anti-knob Fab) (Ad/FGF). Mice were then treated with ganciclovir (GCV) and survival was monitored. Mice treated with retargeted adenovirus had a significant increase in median survival. Ad (10^8 pfu) vs Ad/FGF (10^8 pfu), $p = 0.0025$; Ad (10^9 pfu) vs Ad/FGF (10^9 pfu), $p = 0.007$.

FGF2 retargeting than with the untargeted vector at each dose of virus. Second, the survival curve for mice treated with 10^8 pfu of virus with FGF2 retargeting was the same as that for mice treated with 10^9 pfu of untargeted vector. Thus, FGF2 retargeting effected a 10-fold reduction in viral dose for the same therapeutic outcome. These results thus indicate a potential for increasing the clinical utility and therapeutic index of adenoviral vectors by using a retargeting approach.

Successful targeting following intravenous administration of a targeted adenoviral vector has yet to be reported. In this context, one major hurdle to overcome is hepatic uptake of injected virus, which accounts for greater than 90% of the injected dose. Although initially this problem was considered to be due in part to nonspecific uptake related to entrapment of virus in hepatic sinusoids, recent evidence suggests that this is a receptor-mediated phenomenon and therefore it is potentially addressed by modification of native viral tropism. To this end, Zinn and colleagues[35] investigated the *in vivo* distribution of technetium-labeled adenovirus serotype 5 (Ad5) knob. They found that most of it became localized to the liver 10 minutes after injection. Importantly, this localization could be inhibited by prior injection of an excess of unlabeled Ad5 knob, but not by an excess of serotype 3 (Ad3) knob (which binds to a different receptor), indicating the specificity of hepatic uptake. When labeled Ad5 knob was complexed with 1D6.14 Fab before injection, hepatic uptake was markedly reduced, providing further evidence of the receptor-dependent nature of the uptake and a degree of stability of the Fab to knob bond in the bloodstream.

In summary, developments thus far using immunologic retargeting strategies have established several important principles. Modification of tropism was successfully achieved, indicating that true cell-specific delivery is possible. Evidence to date suggests that limitations in gene transfer due to a deficiency in either CAR, αv integrins, or both may be overcome by retargeting. Not only is efficiency of gene delivery with retargeted complexes comparable to that of wild-type vector, but also in many cases it has resulted in substantial improvement in gene delivery, which is itself a worthwhile goal. Retargeted complexes are efficacious *in vivo*, at least in a compartmental context. The use of a retargeted vector has been shown to enhance a therapeutic endpoint, and finally, the limitations of intravenous application of these vectors, imposed by hepatic uptake of virus, appear to be a receptor-mediated phenomenon and may therefore also be overcome by retargeting. The full potential of immunologic retargeting, however, is yet to be defined, and there are certain practical and theoretic limitations to this approach. Large scale production of bispecific antibody conjugates of consistent configuration is difficult when using the heterobifunctional crosslinkers that have so far been reported. Also, clearance of retargeting complexes and activation of the complement system may limit *in vivo* application. Although further protein engineering refinements, such as the fusion protein "adenobody" approach, may address some of these issues, the stability of the targeting complex-virion bond following systemic delivery remains of concern, especially when attempting intravenous administration. Thus, development of another approach, genetic retargeting, is also being pursued.

GENETIC RETARGETING

In view of the practical and theoretic limitations of the immunologic approach to retargeting just mentioned, development of targeted vectors by genetic manipulation of the virus itself has proceeded alongside the immunologic strategies. In addition, the immunologic targeting approach is not likely to be sufficient for application to the controlled replicating viral vector systems being developed to improve gene delivery to malignant tumors. In these systems, precise targeting of both the initial viral dose and the progeny viruses is particularly important and only achievable by genetic modifications.

Based on the knowledge of native viral binding, a rational place to begin in the development of genetically targeted vectors is with the knob domain, and most strategies have so far focused on this region. The question of whether viral tropism could be modified genetically was initially addressed by Krasnykh and colleagues.[36] A chimeric adenovirus containing the serotype 3 knob on the adenovirus 5 fiber shaft and capsid was produced by homologous recombination in 293 cells, using a modification of the shuttle and rescue plasmid technique developed by Graham.[37] Because Ad5 and Ad3 recognize different receptors, the tropism of the chimeric vector could be assessed by blocking infection of cells with an excess of free Ad5 or Ad3 recombinant knob. This confirmed that an 'Ad5' vector possessing Ad3 tropism had been produced. A similar strategy was reported by Stevenson and colleagues[38] who also demonstrated differences in the gene transfer efficiency of various cell lines depending on whether wild-type or chimeric fiber vectors were used.[38] After the initial proof of principle, incorporation of specific targeting ligands was investigated.

When modifying the knob domain, important structural constraints must be addressed. Because fibers must trimerize to allow attachment to the penton base for subsequent capsid formation, any modification of the fiber must not perturb trimerization.[39] Incorporation of a small ligand (gastrin-releasing peptide) at the carboxy terminal of fiber and subsequent generation of trimers of this chimeric fiber were initially reported by Michael and colleagues.[40] It has since been discovered that there are limits to the size of peptides that can be used in this way. Although the limits probably relate to the actual sequence used rather than its length alone, trimerization is much less likely to occur with ligands longer than 25–30 amino acids. On the other hand, deletion of just 1 amino acid from the carboxy terminal leads to failure of trimerization. Despite these limitations, the addition of small peptide ligands may have utility. For example, we recently added a moiety containing 6 histidine residues (6-His) to the knob carboxy terminal, successfully rescued the virus, and confirmed the binding availability of the 6-His moiety using nickel column chromatography. This virus may be used to address concerns about the stability of the bond between adenoviral vectors and immunologic retargeting complexes. Theoretically, using an immunologic retargeting complex containing an anti-6His antibody, followed by the use of nickel MMPP technology, might allow the formation of a stable covalent bond between the virus and the targeting complex.[41] Wickham[42] recently reported a number of carboxy terminal modifications, including one containing an RGD motif (21 amino acids) to target cell surface integrins and one containing seven lysine residues to target cell surface heparan sulfates. Production of virus with longer carboxy terminal additions was also attempted. A virus containing a 32 amino acid peptide for

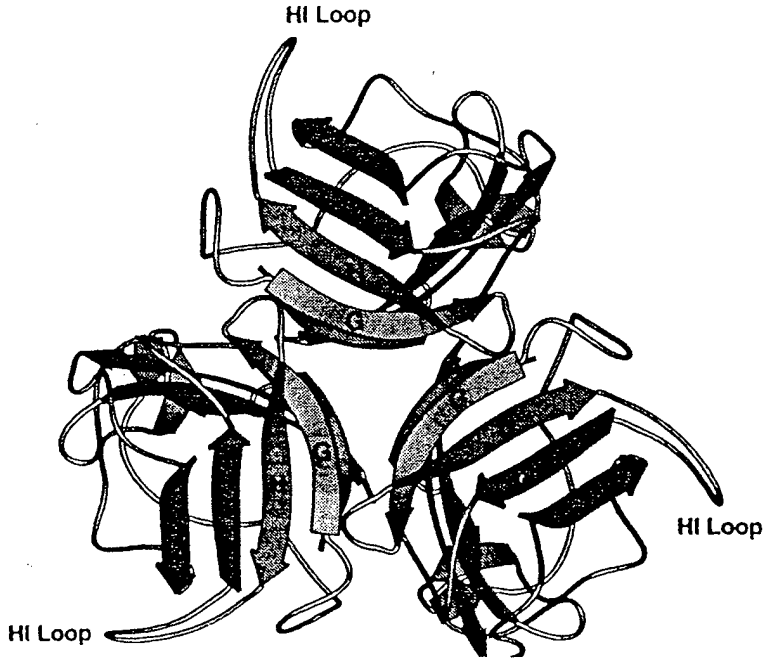

FIGURE 4. The knob trimer showing the position of the HI loop. Reprinted, with permission, from Xia *et al.*, [43] p. 42.

targeting the laminin receptor was produced, but it failed to bind its target receptor, whereas a virus containing a 27 amino acid E-selectin binding peptide could not be produced. Neither of the successfully produced viruses had any modification to native tropism; therefore, their potential application is in the context of enhancing delivery to otherwise poorly transfectable cells, rather than true cell-specific targeting. Nevertheless, enhanced vascular delivery using the polylysine virus was demonstrated *in vivo* in a vascular injury model and may have utility in the therapy of angioplasty restenosis. The RGD-containing virus enhanced gene delivery to endothelial and smooth muscle cells *in vitro*.

In view of the limitations involved in attaching ligands to the carboxy terminal, other regions of the knob may ultimately prove to be better sites for ligand incorporation. In this regard, we investigated the HI loop region of the knob. X-ray crystallographic modeling of the three-dimensional structure of trimeric knob indicates that the HI loop is located on the outer aspect in an area potentially available for interaction with receptors (FIG. 4).[43,44] Also, this region does not appear to be directly involved in trimerization, contains mostly hydrophilic amino acids, and is different lengths in different Ad serotypes, suggesting less rigid structural constraints than at the carboxy terminal. As initial proof of concept we successfully incorporated a FLAG epitope into the HI loop[45] using a technique of recombinant virus generation involving homologous recombination in *Escherichia coli*.[46] Affinity binding to an M2 matrix column confirmed that this epitope was available for binding in the con-

text of the intact virion. Other ligands with more relevant targeting potential have now been incorporated, including a cyclic RGD peptide (which has affinity for tumor vasculature)[47] and somatostatin. The size constraints of ligand incorporation at this site are yet to be determined; therefore, incorporation of large ligands such as EGF and sFvs is currently being investigated. However, it is likely that the sheer size of an sFv will require an alternate strategy such as complete replacement of the entire knob domain.

Ultimately, for true targeting to be achieved, modification to ablate native tropism will need to be addressed. It may be that incorporation of large ligands into the HI loop will simultaneously ablate native tropism by steric hindrance; however, if this is not the case, further modifications will be required. In this regard, receptor binding epitopes within the knob that may be suitable for mutagenesis strategies have been identified by Boulanger.[48] Clearly, if complete replacement of the knob with a targeting and trimerization moiety could be achieved, it would simultaneously ablate native tropism. An integral part of any such strategy will be the use of permissive cell lines possessing the relevant target receptor to allow rescue and propagation of the virus.

Genetic modification strategies are not limited to the fiber. Wickham[49] introduced modifications into the penton base for targeting to cell-specific integrins. Hexon capsid proteins might also be exploited for targeting. An attractive aspect of this approach is the number of hexon proteins, 720, compared to the 36 knob regions; therefore, hexon modification might have the potential for higher affinity binding. Such a strategy, however, will still have to take into account the need to ablate native knob-dependent binding and to address the stoichiometric issues relating to the fiber projecting out from the capsid, to ensure physical accessibility of the ligands introduced into the hexon.

As progress is being made in the development of retargeted vectors, the importance of identifying truly cell-specific ligands has been highlighted. Although there are many established ligands and antibodies that may be candidates in certain settings, in many cases, such as mature airway epithelium, truly specific targets have yet to be discovered; therefore, further target definition is required. This is especially relevant in the context of those genetic retargeting strategies that attempt to target with small peptide ligands. In this regard, the use of bacteriophage panning techniques have shown potential utility in target definition.[47,50,51] Bacteriophage can be engineered to express peptide sequences of various lengths and configurations (e.g., linear or cyclic) on their surface. Libraries of phage can be generated that express all possible sequences of peptide of a defined length or configuration. Using this library we can pan against target proteins, cells, or even organs and tissues *in vivo*. By isolating from the library the phage that has affinity for the target of interest, serial rounds of panning can ultimately identify peptide sequences that show particular affinity for the target. For example, using this approach *in vivo*, Pasqualini and colleagues[52] identified a double cyclic RGD-containing peptide with particular affinity for tumor vasculature. Similar strategies are also being used to define sFvs with targeting potential.

In summary, it has clearly been shown that viral tropism can be modified by genetic strategies. Concurrent ablation of native tropism has not yet been achieved however. The targeting ligands that have been successfully introduced at this stage

are limited to short peptides, and incorporation of larger ligands may require new approaches. Nevertheless, progress in this area has been rapid, and the results to date, coupled with the immunologic retargeting results, indicate that development of a systemically stable, cell-specific vector is a very realistic aim.

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Analysis of Cell-Specific Promoters for Viral Gene Therapy Targeted at the Vascular Endothelium

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Short Title: Endothelium-specific recombinant gene expression.

ABSTRACT

The use of viral vectors for vascular gene therapy targeted at the endothelium is limited by the promiscuous tropism of vectors and non-specificity of viral promoters, resulting in high-level transgene expression in multiple tissues. In order to evaluate suitable endothelial cell (EC)-specific promoters for vascular gene therapy, we have directly compared the ability of the *fms*-like tyrosine kinase-1 (FLT-1), intercellular adhesion molecule-2 (ICAM-2) and von Willebrand factor (vWF) promoters to drive EC-restricted transcription following cloning into adenoviral vectors upstream of *lac*Z. Vastly different expression profiles were observed. While both FLT-1 and ICAM-2 promoters generated transgene expression levels similar to CMV in ECs *in vitro*, vWF expression levels were extremely low. Analysis of non-EC types revealed that ICAM-2, but not FLT-1, evoked leaky transgene expression thus identifying FLT-1 as the most selective promoter. Using an *ex vivo* human gene therapy model, the FLT-1 promoter demonstrated EC-specific transgene expression in intact human vein but no detectable expression from infected exposed smooth muscle cells in endothelial cell-denuded vein. Furthermore, when adenoviruses were systemically administered to mice, the FLT-1 promoter demonstrated extremely low-level gene expression in the liver, the major target organ for adenoviral transduction *in vivo*. This study highlights the potential of using the FLT-1 promoter for local and systemic human gene therapy in hypertension and its complications.

Keywords: adenovirus, promoter, gene therapy, endothelium, FLT-1, vWF, ICAM-2

The vascular endothelium is an attractive target for clinical gene therapy. Its proximity to circulating blood and the vessel wall throughout the body underlies the need to generate gene therapeutic vehicles aimed specifically at the endothelium. The endothelium plays a fundamental role in many vascular pathologies including endothelial dysfunction associated with hypertension and early atherogenesis, plaque rupture, post-angioplasty restenosis and late vein graft failure. However, current gene delivery vectors, both viral and non-viral, demonstrate poor selectivity to endothelium following local or systemic delivery. There is therefore an urgent need to develop endothelial cell-specific vascular gene transfer vectors, which would be applicable to prevention or attenuation of vascular complications of hypertension

We have recently demonstrated the ability to re-target adenoviral tropism to endothelial cells resulting in high-level and selective targeting at the level of virus-cell interaction.[1] While this results in endothelial cell-specific infection with adenoviral vectors, the inclusion of cell-specific promoters would further enhance selectivity and hence safety. Transgene production by the use of viral promoters such as the cytomegalovirus immediate early promoter (CMV) or the Rous sarcoma virus (RSV) evoke high-level gene expression in all cell types transduced, which clearly may be deleterious in clinical situations. The promoters for a number of genes whose transcription is restricted to endothelium have been sequenced and partially characterised, these include: vascular cell adhesion molecule-1 (VCAM-1),[2] endothelial nitric oxide synthase (eNOS),[3] vWF,[4] FLT-1,[5] tyrosine kinase with immunoglobulin and epidermal growth factor homology domains (TIE),[6] kinase-like domain receptor (KDR)[7] and ICAM-2.[8] To date, no studies have directly compared candidate EC-specific promoters within the same viral gene delivery system. Here we document the gene expression profiles of adenoviral vectors using the candidate vWF, FLT-1 and ICAM-2

promoters *in vitro*, *ex vivo* and *in vivo*.

METHODS

Cell culture. 293 cells, human primary foreskin fibroblasts (used between passages 3 and 6, a generous gift from Dr. Mark Bond, Bristol Heart Institute, University of Bristol, UK), HeLa and HepG2 cell lines were maintained in Minimal Essential Media (MEM) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L L-glutamine and 10% (v/v) fetal calf serum (FCS). Human umbilical vein endothelial cells (HUVEC) and human saphenous vein endothelial cells (HSVEC) were isolated by a modified version described by Jaffe *et al*[9] and used below passage 5. Endothelial cells were identified by immunofluorescence for vWF. Vascular smooth muscle cells (VSMC) were from medial explants of human saphenous vein obtained from patients undergoing bypass surgery.[10] VSMC were cultured until first passage in SmGM BulletKit media (Clonetics, San Diego, CA., USA). At passage 1 they were confirmed as VSMC by immunofluorescence for SMC α-actin. For subsequent passages, VSMC were cultured in DMEM (4500 mg/L glucose, glutamax-1) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 20% (v/v) FCS. The IP-IB murine SV-40 transformed endothelial cell line (American Type Tissue Culture collection) were maintained as for VSMC except with 10% FCS. All cells were maintained at 37°C under a mixture of 95% air and 5% CO_2.

Adenoviral constructs. The adenoviruses RAdCMV[11] and RAdLUC (obtained from Robert Gerard) express the bacterial *Lac*Z and firefly luciferase genes respectively under the control of the CMV promoter. PCR was used to clone endothelial cell-specific promoters for FLT-1 (-748 to +284),[5] ICAM-2 (-367 to –34)[8] and vWF (-487 to +247)[4] into adenoviral vectors. Oligonucleotide primers were designed spanning the above sequences (FLT-1 sense 5' CCC GCA TGC CTT CTA GGA AGC AGA AGA CTG AGG A 3', antisense 5' CCC TCT AGA GTG AGC GCG ACG CGG

CCT GCT CGC C 3'), (ICAM-2 sense 5' CCA TGG GAT TTG GGG TTC CC 3', anti-sense 5' CCA AGG GCT GCC TGG AGG GA 3') and (vWF sense 5' CCC GCA TGC ATC TTT AGC CGA TCC ATT CAA CCC T 3', antisense 5' CCC TCT AGA CCC CTG CAA ATG AGG GCT GCG GCT A 3'). An *Sph*I site and clamp (underlined) was synthesized at the 5' end of the FLT-1 and vWF sense primers and an *Xba*I site and clamp (underlined) at the 5' end of each FLT-1 and vWF antisense primer to create unique *Sph*I and *Xba*I cloning sites. For ICAM-2, a *Nco*I site was engineered at the 5' end of the sense primer. For amplification of vWF, 1 ng HUVEC DNA template was amplified using *Vent* DNA polymerase (New England Biolabs, UK.), for 35 cycles at 94°C for 1 minute, 54°C for 1 minute and 72°C for 1 minute. FLT-1 was amplified from 1 ng plasmid DNA template (a gift from L. Williams, UCSF, USA.) using the same conditions as for vWF except annealing at 56°C. ICAM-2 was amplified from human genomic DNA using annealing at 61°C. FLT-1 and vWF promoters were cloned into the *Sph*I/*Xba*I site of pMV10[11] upstream of *Lac*Z and the CMV polyadenylation signal. Sequencing confirmed that no PCR induced mutations were present. Expression cassettes were excised and cloned into the *Hin*dIII site of the adenovirus shuttle pMV60. ICAM-2 was cloned into pGEM-T-Easy (Promega, UK) and sequenced. The fragment was then sub-cloned as a *Hin*DIII-*Sma*I fragment and transferred into pΔE1sp1B (Microbix Biosystems, Canada) with the *lac*Z gene excised from pCA17 (Microbix Biosystems) and confirmed by sequencing. Recombinant adenoviruses were generated by homologous recombination with pJM17 in low passage 293 cells[12]. Recombinant adenoviruses designated RAdFLT-1, RAdvWF and RAdICAM-2 were plaque-purified, propagated on 293 cells, caesium chloride-banded and titered using standard techniques.[13] Recombinant adenoviruses were assessed

for lack of replication-competent adenovirus by plaque titration on non-permissive HeLa cells and immunofluoresence for E1a following infection of HeLa.

Infection protocols. Cells were trypsinised and plated into 24 well plates at 5 x 10^4 cells/well (2.5 x 10^4 for IP-IB cells). Immediately prior to infection an accurate cell count was determined. We used a dose range to assess β-galactosidase production in each cell type tested. Due to differing levels of both adenovirus cell entry receptors, $\alpha_v\beta_{3/5}$ integrins and the Coxsackie/ adenovirus receptor (CAR), on different cell types, initial experiments were performed in order to allow direct comparison of each promoter. The quantity of virus required to achieve 100% infection into each cell type was first determined using the constitutive viral promoter in RAdCMV. Once this optimal level was determined, transgene levels were further characterised at 50% and 10% infection for each cell type. HUVEC, HSVEC and IP-IB were infected with 100, 500 and 1000 pfu/cell of each recombinant adenovirus in triplicate cultures, VSMC with 60, 300 or 600 pfu/cell, fibroblasts with 30, 150 or 300 pfu/cell and HepG2s and HeLa with 10, 50 or 100 pfu/cell. A media change was carried out 16 hours post-infection. Cells were incubated for a further 48 hours in complete media before either fixing and staining with X-gal stain (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [100 mmol/L sodium phosphate, pH 7.3 (77 mmol/L Na_2HPO_4, 23 mmol/L NaH_2PO_4), 1.3 mmol/L $MgCl_2$, 3 mmol/L $K_3Fe(CN)_6$, 3 mmol/L $K_4Fe(CN)_6$ and X-gal (20 mg/ml)] as described,[14] or harvesting for quantification (see below). All photomicrographs were taken randomly.

Quantitative β-galactosidase assay. β-galactosidase production was quantified using a chemiluminescent reporter gene assay (Galacto-Light Plus, Tropix, MA, USA). 48 hours post-infection cells were lysed for 10 minutes at 4°C in 50 μl of lysis buffer. 2-20 μl of each resulting

cell lysate was analysed for β-galactosidase levels according to the manufacturer's recommendations. Each sample was quantified within the linear range of a standard curve.

***In situ* infection of human saphenous vein.** Freshly isolated (EC-intact) and surgically prepared (EC-denuded) vein segments were obtained from patients undergoing bypass surgery, collected and prepared as described.[15,16] Surgically prepared vein segments that had undergone manual distension, were obtained after storage in patients' heparinised blood for 60 -120 minutes. Freshly isolated vein was obtained after removal from the patient with minimal handling and prior to distension. Vein segments were cannulated and infected with 120 μl adenovirus (1.2×10^{10} pfu/ml) as described.[17] This protocol results in 39±7% transduction of exposed lumenal surface cells[17]. Veins were pinned out lumenal surface uppermost and cultured for 7 days in complete culture media (RPMI 1640, 100 IU/ml penicillin, 100 μg/ml streptomycin, 8 μg/ml gentamicin, 2 mmol/L L-glutamine and 30% FCS).[15,16] β-galactosidase production was assessed at day 7 by X-gal staining and frozen serial sections.

***In vivo* analysis of promoter activity.** Athymic nude mice (female, 8 weeks old; Frederick Cancer Research) were injected with 5×10^{10} particles of RAdCMV, RAdFLT-1 or RAdLUC (negative control) in 200 μl of PBS. 3 days post-infection livers were harvested, frozen and ground to a fine powder using a pestle and mortar. Lysed extracts were assayed for β-galactosidase activity using Galacto-light Plus according to the manufacturer's recommendations.

Statistical analysis. All data were analysed using an unpaired Students' t test and are shown as the mean value ± the standard error of the mean (SEM).

RESULTS

Analysis of β-galactosidase production *in vitro*. To evaluate the ability of RAdFLT-1, RAdICAM-2 or RAdvWF to drive cell-specific gene expression, histological analysis of β-galactosidase production from each adenovirus was performed. As expected primary HUVEC, HSVEC, VSMC and fibroblast cells required higher titers than HepG2 and Hela cells (Figure 1). Infection of cells with RAdFLT-1, RAdICAM-2 or RAdvWF, however, produced vastly different β-galactosidase staining compared to that from RAdCMV. In HUVEC and HSVEC RAdFLT-1 and RAdICAM-2, but not RAdvWF, produced high-levels of β-galactosidase, comparable to that evoked by RAdCMV (Figure 1). Evaluation of primary VSMC and human fibroblasts demonstrated that RAdFLT-1 and RAdvWF produced lower levels of β-galactosidase positive cells compared to RAdCMV (Figure 1). However, RAdICAM-2, unlike RAdFLT-1 and RAdvWF, appeared to evoke high-level transgene expression in VSMC, Hela, fibroblasts and HepG2 cells (Figure 1).

We next quantified β-galactosidase levels in cell extracts from both EC (Figure 2) and non-EC (Figure 3). In ECs, RAdFLT-1 and RAdICAM-2 produced high-levels of β-galactosidase. Interestingly, RAdICAM-2 evoked levels significantly higher than those from CMV (Figure 2). In accordance with histological analysis, vWF as expected, induced very low levels of β-galactosidase in both HSVEC and HUVEC (Figure 2). Analysis of non-ECs revealed divergent results from each promoter (Figure 3). RAdICAM-2 was extremely active in VSMC, Hela, fibroblasts and HepG2 cells but levels from RAdFLT-1 were significantly lower than CMV (Figure 3). In similarity to EC, vWF demonstrated low-level activity in non-EC types although some β-galactosidase was observed in VSMC (Figure 3). These data demonstrate that RAdFLT-1 induces high-level and selective EC-specific gene expression *in vitro* and was therefore selected for *ex vivo* and *in vivo* experiments.

Promoter activity in human saphenous vein *ex vivo*. We further sought to define whether RAdFLT-1 could evoke similarly high-level EC-specific transcription in a clinically appropriate human model, in which reporter gene expression can be evaluated in both endothelial (undamaged, freshly isolated vein) and non-endothelial (endothelial-denuded, surgically-prepared vein) cell types. As expected, localised lumenal-specific delivery of RAdCMV to human saphenous vein demonstrated widespread lumenal surface staining of cells for β-galactosidase in both freshly isolated and surgically prepared human saphenous vein (Figure 4). Histological cross sections revealed transgene expression at the lumenal surface of both vein types (Figure 4). Conversely, RAdFLT-1 demonstrated widespread staining in freshly isolated saphenous vein, but minimal lumenal surface staining in surgically prepared vein (Figure 4). Analysis of histological sections demonstrated this selective expression profile with substantial staining in freshly isolated, but no staining in surgically-prepared vein (Figure 4). This demonstrates the ability of FLT-1 to evoke high-level EC-specific gene expression following local delivery into human vein.

Analysis of promoter activity *in vivo*. For many vascular gene delivery protocols based on both local or systemic delivery approaches, a potential deleterious effect of the transgene on non-target tissue may be apparent if the vector has access to its primary site(s) of infection, either through leakage (in the case of local delivery) or through the bloodstream (systemic delivery). Therefore, we evaluated the activity of the FLT-1 and CMV promoter in liver *in vivo* as this is the primary site for adenoviral infection. We first defined that the human FLT-1 promoter was functional in murine endothelial cells. Although significantly less, RAdFLT-1 activity in IP-1B cells was approximately 40% of that produced by RAdCMV demonstrating functional FLT-1 activity in murine endothelial cells (Figure 5A). We next evaluated β-galactosidase levels in the livers of mice injected with

RAdCMV, RAdFLT-1 or RAdLUC (negative control). As expected levels of β-galactosidase were very high in livers of mice injected with RAdCMV, livers from RAdFLT-1-injected mice, however, demonstrated extremely low levels of β -galactosidase production (Figure 5B).

DISCUSSION

Effective use of gene therapy vectors within clinical settings requires the development of suitable targeting strategies. One of the major challenges is to develop vectors incorporating targeting both at the level of vector-cell interaction and at the level of transcription. In this study we directly compared, using an adenoviral system, transcription from 3 candidate endothelium-specific promoters in isolated cells, in a relevant human vein model and *in vivo*. We demonstrated high-level reporter gene expression in human endothelial cells from the FLT-1 and ICAM-2 promoter but not the vWF promoter. However, while expression from the FLT-1 promoter was restricted to endothelial cells, the ICAM-2 promoter was extremely leaky. The FLT-1 promoter also drove high-level, endothelial cell-restricted expression in human vein following local delivery and did not demonstrate any activity in hepatocytes *in vivo* following systemic delivery. We were surprised that the activity of the vWF promoter in endothelial cells was extremely low. Original characterisation of this promoter using plasmid-based vectors demonstrated that expression levels in bovine EC from the –487/+247 bp vWF fragment were 90% of the level achieved with the core fragment (-90 to +155 bp).[4] In non-EC types, the core promoter fragment achieved similar levels in bovine SMC and Hela cells to that observed in EC, but the levels from the -487/+247 bp fragment were negligible.[4] However, we demonstrate that expression levels from the -487/+247 bp vWF promoter are significantly lower than other candidate promoters when engineered in to adenoviral vectors, presumably due to the distinct influences exposed to within adenoviral vectors, as has been observed for the human ventricular/slow myosin light chain 1 promoter.[18] The 334 bp ICAM-2 promoter used in this study contains two GATA motifs, three ETS, one SP1 and one CACCC,[8] which compares favourably with the structure of the mouse ICAM-2 promoter (one GATA motif

and two ETS motifs oriented as the human promoter and with a CACCC motif).[19] We observed that this promoter drove high-level gene expression in endothelial cells and in non-EC types. These results differ to those published.[8,20] In transgenic animals, the same ICAM-2 promoter demonstrated high-level consistent transgene expression in EC of all blood vessels in the heart, kidney, lung, liver and pancreas with negligible expression in other cell types except neutrophils and monocytes.[8] A further study using the same fragment in plasmid vectors showed high level activity in bovine EC, but not in COS cells although no human EC were tested.[20] Therefore, importantly and in similarity to vWF, when re-engineered into viral vectors ICAM-2 promoter activity and selectivity is markedly altered. While our data has demonstrated the limitations of using the vWF and ICAM-2 promoters for endothelium-specific gene transcription in adenoviral systems, it is clear that the findings from isolated cell cultures cannot be directly compared to previous *in vivo* findings using other gene transfer and transgenic systems.

Transgene expression *in vitro* from the FLT-1 promoter was very high and selective to EC in agreement with the study by Morishita *et al.*[5] who analysed expression in isolated cells *in vitro*. Direct comparison with the vWF and ICAM-2 promoters confirmed the potential for this promoter. We extended these findings to include relevant models and demonstrated that the FLT-1 promoter may be useful clinically for vascular gene therapy. In evaluating the EC specific activity of the FLT-1 promoter in human saphenous vein we demonstrated potential clinical utility for FLT-1 when delivered locally into human vessels for delivery of therapeutic genes such as metalloproteinase inhibitors.[17,21] Furthermore, it is clear that systemic dissemination of virus may have deleterious consequences, particularly for pro-death or pro-angiogenic genes.[22-26] We found that FLT-1 activity in hepatocytes *in vitro* and *in vivo* was extremely low indicating that if vector

dissemination occurred during local delivery to the vessel wall, the use of FLT-1 would avoid undesirable transgene expression in the liver.

As our *in vitro* and *ex vivo* experiments were performed in the presence of serum it is clear that cells will be at different stages in the cell cycle and this will vary considerably between different cell types. It will therefore be important to define promoter activity in cells were activity is observed based on transgene expression and cell cycle characteristics. Furthermore, in the context of human saphenous vein it will be important to document FLT-1 promoter activity in quiescent and damaged endothelium when considering endothelial-restricted gene expression in coronary artery bypass grafts. In summary, we have demonstrated the ability of the FLT-1 promoter to drive EC-restricted expression *in vitro* and in human vein *ex vivo*. Furthermore, FLT-1 was shown to be inactive in hepatocytes following systemic delivery into mice *in vivo*. This data clearly identifies FLT-1 as a candidate EC-selective promoter for gene therapy protocols targeting the endothelium using both local and systemic delivery approaches. The ability to target the endothelium provides the first step towards refined local and systemic gene transfer in hypertension and its complications.

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LEGENDS TO FIGURES

Figure 1. β-galactosidase staining in isolated endothelial and non-endothelial cells. Phase contrast pictures of cells stained for β-galactosidase expression following infection with RAdCMV, RAdFLT-1, RAdvWF or RAdICAM-2 (bottom panel). Primary human VSMC (300 pfu/cell), primary human fibroblasts (100 pfu/cell), HepG2 hepatocytes (100 pfu/cell), HeLa (100 pfu/cell), primary HUVEC (500 pfu/cell) or primary HSVEC (1000 pfu/cell) are shown. Representative of three independent experiments each performed in triplicate.

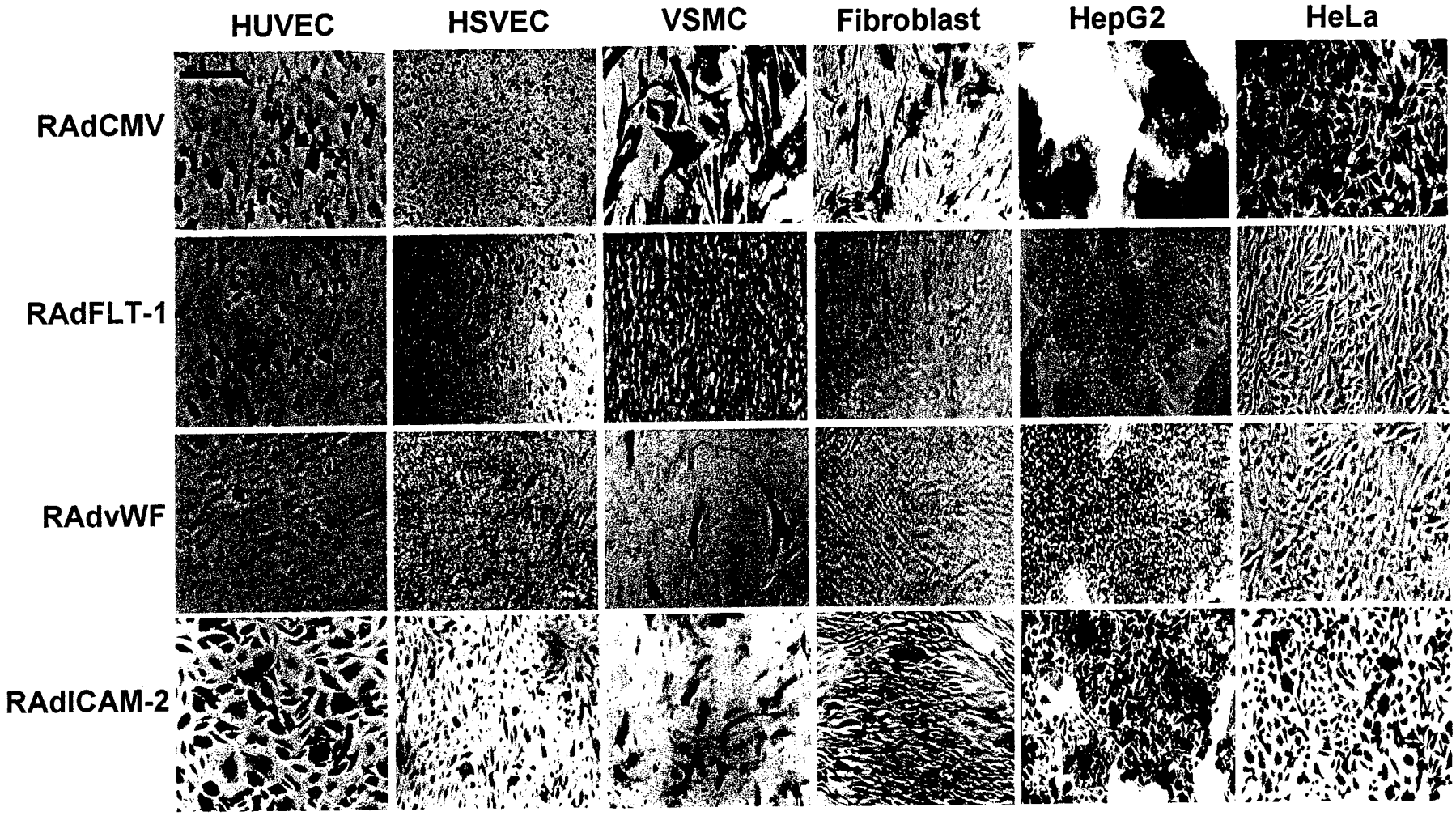
Figure 2. Promoter activity in primary human EC. β-galactosidase was quantified in cell lysates from primary endothelial cells HUVEC and HSVEC infected with RAdCMV, RAdFLT-1, RAdvWF or RAdICAM-2 at the MOI shown. * indicates statistical significance vs. CMV at the same MOI ($p < 0.05$). Data are representative of 3 independent experiments.

Figure 3. Promoter activity in non-EC. β-galactosidase was quantified in cell lysates from primary non-endothelial cells including VSMC, dermal fibroblasts and non-endothelial cell lines HepG2 and Hela infected with RAdCMV, RAdFLT-1, RAdvWF or RAdICAM-2. * indicates statistical significance vs. CMV at the same MOI ($p < 0.05$). Representative of 3 independent experiments.

Figure 4. CMV and FLT-1 promoter activity in human saphenous vein *ex vivo*. (A, B, E, F) *en face* β-galactosidase staining of isolated segments of freshly isolated (A, E) or surgically prepared (B, F) human saphenous vein 7 days post-lumenal specific infection. (C, D, G, H) show representative frozen cross sections of freshly isolated (C, G) or surgically prepared (D, H) vein. The scale bar in C represents 25 μm and is applicable to panels C, D, G and H. Examples of β-

galactosidase positive cells are indicated with arrows. Representative of 6 independent experiments/treatment.

Figure 5. FLT-1 promoter activity in murine EC *in vitro* and liver *in vivo*. (A) FLT-1 and CMV activity in the mouse EC-line IP-1B. Representative of 3 experiments (B) β-galactosidase production in murine liver tissue extracted 3 days post-infection (n=5 mice/promoter). *$p<0.05$ vs. CMV.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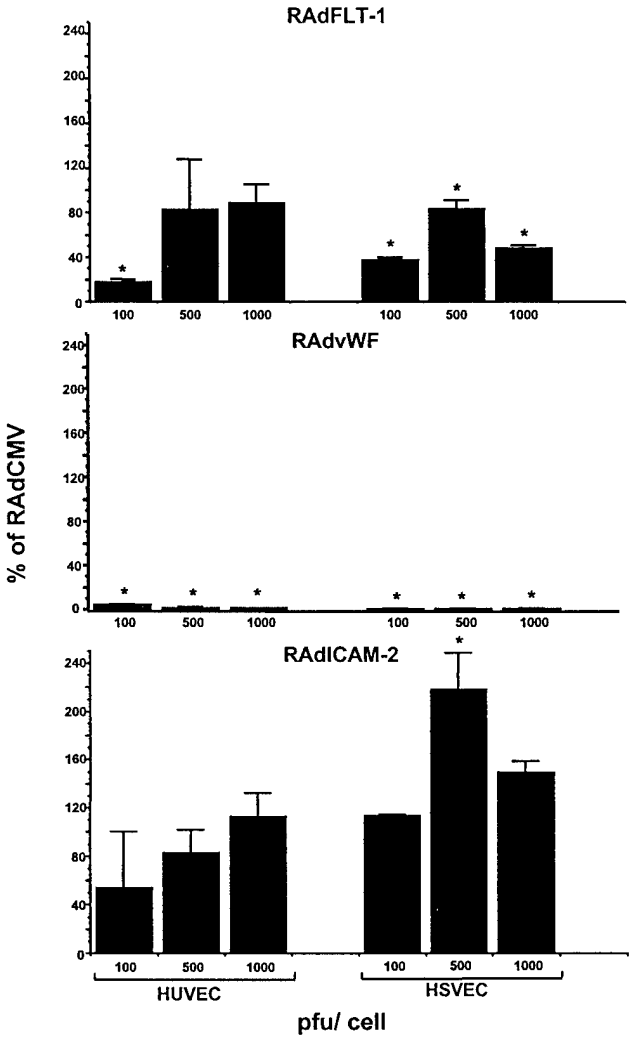
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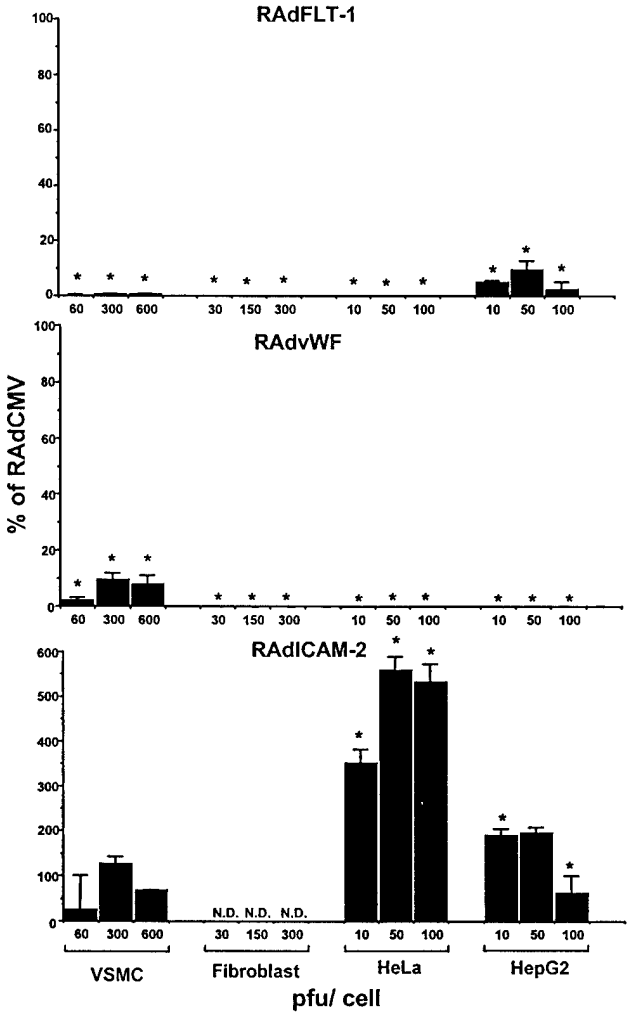
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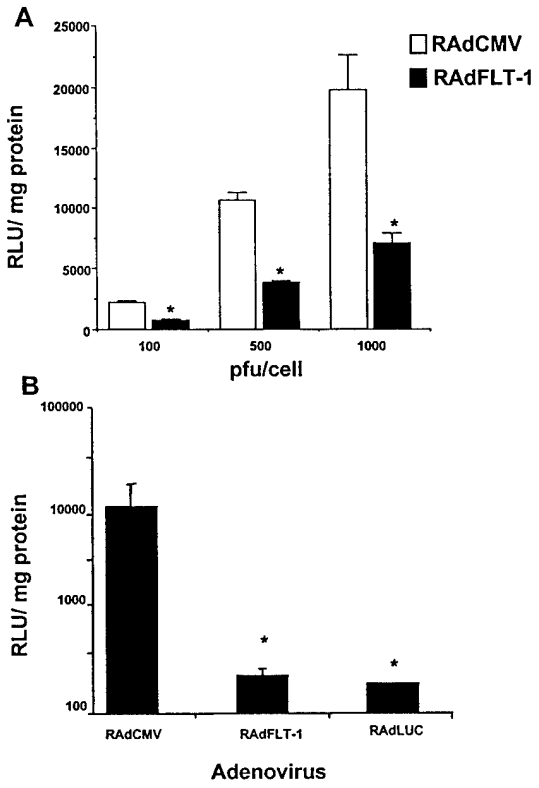
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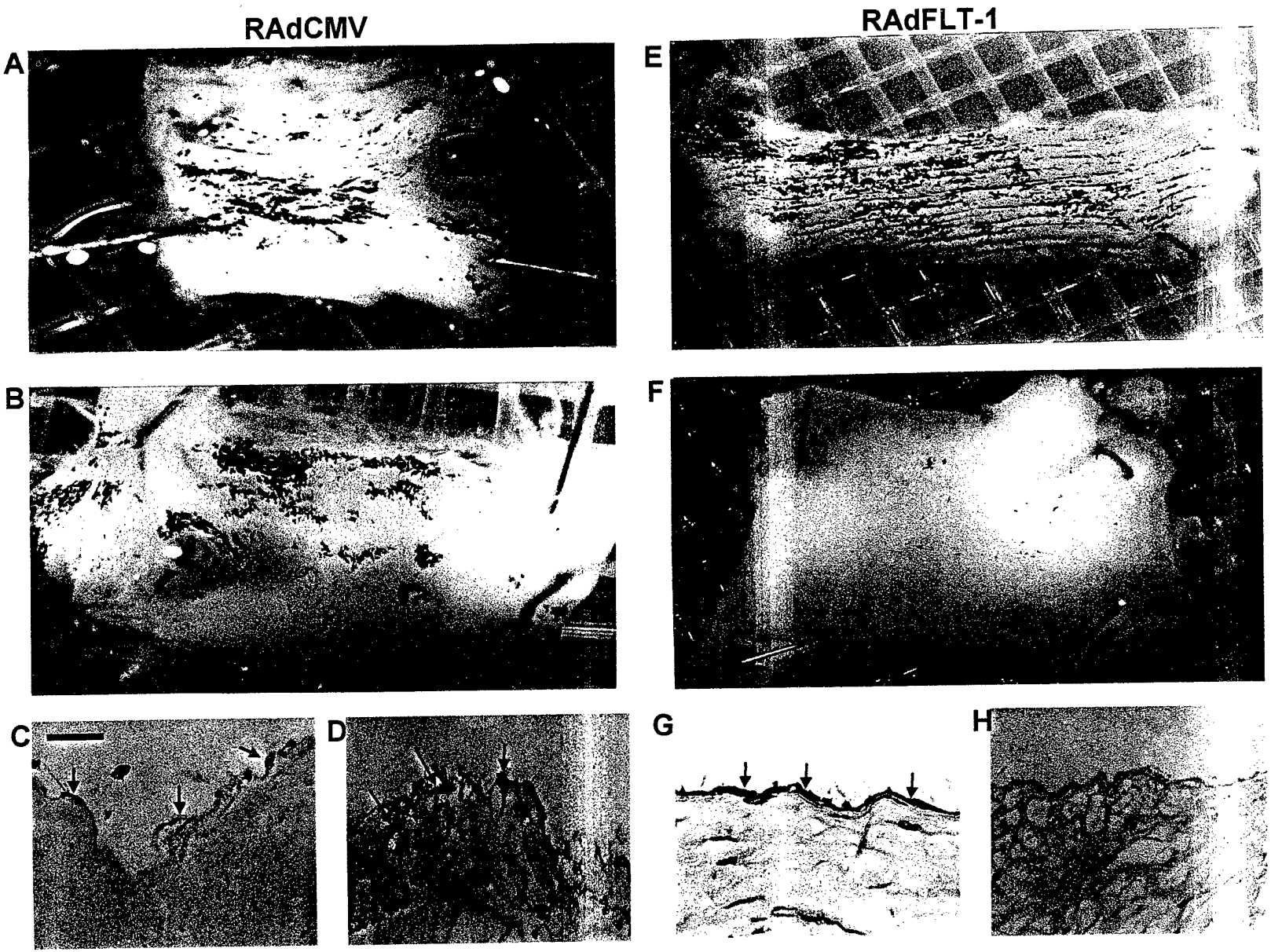
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Targeted Vectors for Cancer Gene Therapy

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I. Introduction

A. Gene Therapy for Cancer

The genetic fingerprint associated with the malignant transformation and progression, consisting of a myriad of acquired genetic lesions, is now being identified rapidly and precisely in a large variety of human cancers. Armed with this knowledge of the molecular anatomy of the cancer cell, gene therapy has emerged as a rational method of therapeutic, and possibly preventive, intervention against cancer targeted at the level of cellular gene expression.

B. Why Targeted Vectors Are Needed For Cancer Gene Therapy

Gene therapy attempts to alter the cancerous pathophysiologic state by delivering nucleic acids into tumoral or normal cells. The expression of transferred genes may achieve a desired effect on the cellular phenotype. Based on this concept, a number of strategies have been developed to accomplish cancer gene therapy. These approaches include: (*i*) mutation compensation, (*ii*) molecular chemotherapy, (*iii*) genetic immunopotentiation, and (*iv*) viral oncolysis. For mutation compensation, gene therapy techniques are designed to correct the molecular lesions that are etiologic of malignant transformation, or to avoid the contribution to the malignant growth by tumor-supporting, non-malignant stromal cells. For molecular chemotherapy, methods have been developed to achieve selective delivery or expression of a toxin gene in cancer, or stromal cells, to induce their eradication, or alternatively to increase their sensitivity to concomitant chemotherapy or radiotherapy. Also, attempts are made to deliver genetic sequences that protect normal bone marrow cells from the toxic effects of standard chemotherapeutic drugs, thus allowing the administration of higher drug doses without reaching otherwise limiting myelosuppression. Genetic immunopotentiation strategies attempt to achieve active immunization against tumor-associated antigens by gene transfer methodologies. Both tumor cells and cellular components of the immune system have been genetically modified to this end. Viral oncolysis is a means to induce tumor cell eradication by exploiting the lytic life cycle of replication-competent viruses.

entry into the cell is available, however, thus permitting the evaluation of a variety of approaches for viral targeting that might satisfy the aforementioned requirements.

5. The Treatment Of Disseminated Neoplasms Requires Targeting The Tumor And Bypassing Several Physiological Barriers

A majority of cancer patients are diagnosed when their tumors have already disseminated systemically. The use of gene therapy strategies targeted directly against the tumor would then require that the vector system is capable, after intravascular administration, of both systemic distribution into the multiple organ sites that harbor cancer foci and of efficient and selective gene delivery into target cancer cells thereafter. Although there exist a reasonable understanding of the native determinants of viral tropism at the cellular level (see below), a scarcity of information is, however, apparent regarding the determinants of vector efficacy at the entire organism level, which seem in fact distinct from the former. The physical properties and stability of viral particles in the presence of the complement, their sequestration by cells of the mononuclear phagocytic system, including liver Kupffer cells, and the structure of the vascular endothelium and dynamics of vascular egression are all being analyzed in this context. Certainly, a more complete understanding of these and related immunological factors (neutralizing antibodies and cellular immune response) may allow developing targeting strategies and, thus, progressing in the utilization of viral vectors *in vivo*.

II. Modalities of Vector Targeting

Targeted gene therapy for cancer can be accomplished at different levels. In one approach, the spatial distribution of the vector is restricted by virtue of the maneuver used for its administration into the body, which physically confines the vector into a site or cavity. In addition, targeting can be based on exploiting the distinctive physiology of solid tumors. Alternatively, the tumor cell can be targeted at the level of transduction to achieve the selective delivery of the therapeutic gene. This involves the derivation of a vector that binds selectively to the target cancer cell. Lastly, the therapeutic gene can be placed under the control of tumor-specific transcriptional regulatory sequences that are activated in tumor cells but not in normal cells and therefore target expression selectively to the tumor cell (**Table 1**). Of note, as mentioned above, these modifications of viral vectors are implemented not only for restricting the host-range, but also for enhancing the capacity for gene transfer to certain cells and for extending the types of cells that can be infected effectively.

A. Physical Targeting

The feasibility of the first human clinical trials of gene therapy was predicated upon the capacity to efficiently infect T cells with recombinant retroviral vectors. This was made possible only by removing the target cells from the patient, appropriately maintaining and modifying genetically the cells in the test tube, and by later infusing them back into the patient. In this case, cell targeting was obtained by cell isolation. Unfortunately, this approach is useful only with sessile cells, when the cells are aimed for generating an immune response, or when the cells secrete a therapeutic product. In another early example of physical targeting, plasmid DNA encoding the costimulatory gene B7 was injected directly into melanoma nodules to generate an antitumoral immune response. This method of gene delivery, however, is inefficient for most purposes, and does not precisely spare non-tumor cells from gene transfer. These two means of targeting, albeit rudimentary, have proved however the most effective to date.

More recently, attempts have been made to increase the delivered vector inoculum by confining the vector administration to closed anatomical compartments, such as the

pleural and peritoneal cavities, frequently affected by malignancies of regional distribution. The levels of gene transfer achieved with retroviral and adenoviral vectors in several clinical trials in mesothelioma and ovarian cancer, though, have been insufficient to translate into clinically relevant effects. In addition, infusion of vectors into the vasculature of target organs has been explored, mainly as a means to allow delivery of higher vector doses and, thus, to increase efficiency while simultaneously decreasing exposure of non-target tissues to the vector. In fact, vector-associated toxicities have been prohibiting in animal models and humans, such as those seen after intra-hepatic artery infusion of adenoviral vector, thus revealing a small therapeutic index. These observations highlight the need of vector tropism-modifications, perhaps via exploitation of the tumor physiology and via improved vector design.

B. Targeting Strategies Exploiting Tumor Physiology

1. Tumor Cells Divide in A Background of Quiescent Cells

The physiology of solid tumors at the microenvironmental level provides a unique target for making cancer treatment more selective. Early in the development of vector systems, virologists observed the dependence of retroviral infection on cell division, and proposed to exploit this feature to selectively deliver genes into the dividing tumor cells, surrounded by quiescent non-tumor cells. Unfortunately, although feasible with highly proliferative murine tumor models, this approach is suboptimal for human tumors, in which the fraction of actively dividing tumor cells is usually less than 20% at any given time point. In addition, all other viral vectors in current use do not require cells to divide for gene transfer and transgene expression to occur, thus limiting the value of exploiting this aspect of tumor biology for targeting purposes.

Recently, viral vectors have been engineered for allowing their selective replication in tumor cells. In some cases, the cell phenotype that determines the desired selectivity is associated with cell proliferation. This fact, for the reason mentioned above, might restrict the replication and compromise the potency of such replicative viral vectors. It would seem preferable that, for the sake of potency, selective replication is engineered based upon more widespread, nearly universal tumor cell traits.

2. The Tumor Microenvironment is Hypoxic

The regions of hypoxia and necrosis within solid tumors present opportunities for targeted, tumor-selective gene therapy. For example, hypoxia induces the transcription of certain genes, such as VEGF and erythropoietin, via the activity of hypoxia responsive elements (HRE) located at the 3' flanking region of those genes. Gene therapy strategies have been evaluated whereby hypoxia induces the transcription of a prodrug-activating enzyme gene controlled by a HRE. These hypoxia-inducible vectors have been shown to drive indeed focal transgene expression in murine tumor models. It remains to be seen, however, whether hypoxia can be exploited in the microenvironment of human tumors, in which a typically smaller growth fraction and perhaps a more heterogeneous distribution of hypoxic foci will impose more challenging conditions for efficient targeting.

Gene therapy strategies could similarly be designed to exploit tumor necrosis. In this regard, certain species of anaerobic bacteria of the genus *Clostridium* can selectively germinate and grow in hypoxic/necrotic regions of solid tumors after intravenous injection of spores. Thus, it might prove possible to exploit clostridia as gene therapy vectors engineered to express therapeutic genes, e.g. a prodrug-activating enzyme. Studies in murine tumor models suggest the feasibility of this concept.

3. The Tumor Vasculature is Aberrant

Tumors require constant new vessel growth to maintain their local progression, invasion, and metastasis. The resulting vasculature, however, develops structural and physiological abnormalities that render the vessels leaky and the blood circulation sluggish and irregular. These characteristics determine obstacles for many therapeutic interventions, but also offer some opportunities. For instance, the increased vascular permeability, due to incomplete endothelial cell and basal membrane lining, has been shown to allow the targeting of anticancer drugs into tumors via small, sterically stabilized (stealth) liposomes with prolonged bioavailability. Alternatively, angiogenic endothelial cells have been shown to avidly bind and internalize cationic liposomes and liposome-DNA complexes (but not other types of liposomes), thus raising the possibility

of using liposomes to deliver therapeutic genes selectively to the endothelium at areas of angiogenesis, rather than to the tumor or interstitial cells. This would possibly be used to exert an anti-angiogenic effect, and an anti-tumoral outcome would conceivably ensue.

4. The Carcinogenic Transformation is Associated with a Lower Threshold For Apoptosis

Tumor cells are characterized by a lower threshold than normal cells for undergoing apoptosis after exposure to certain pro-apoptotic stimuli. Although not yet fully understood, this differential sensitivity offers a potential therapeutic window for gene-based pro-apoptotic interventions. Given the ubiquity of the numerous cellular proteins involved in regulating apoptotic pathways, however, highly selective activation of the lethal processes in cancer cells might still be a critical requirement of therapeutic maneuvers. In that case, targeted gene transfer, as described below, could restrict the expression of pro-apoptotic genes to the tumor cells.

C. Transductional (Cell Surface) Targeting

Attempts to modulate the binding of vectors to cell surface receptors have consisted most frequently on modifying viral vectors, given their generally higher gene transfer efficiency. In this regard, the ability to alter viral binding tropism has followed the increasing understanding of the basic biology of viral entry. Thus, knowledge on retrovirus cell entry led to the first maneuvers for viral vector targeting using recombinant retrovirus, rapidly followed for analogous strategies with recombinant adenovirus and later with adeno-associated virus, herpesvirus, and others as soon as the corresponding viral entry biology has been defined.

1. Biology of Viral Entry Into Target Cells

In general, viral entry into target cells occurs in three phases. *First*, viruses adsorb to a receptor in the cell surface. Adsorption occurs via molecular interactions of viral surface proteins with molecules in the cell's plasma membrane. This process proceeds as well

at 37C and at 4C. For example, the surface subunit of the lentivirus envelope glycoprotein binds to CD4 and the chemokine receptors; the herpes simplex virus envelope glycoproteins bind to heparan sulfate and to a tumor necrosis factor receptor; and the adenoviral carboxy-terminal knob domain of the fiber bind to the coxsackievirus and adenovirus receptor, CAR. *Second*, viruses penetrate through or fuse with the cell membrane and are uncoated as they enter the cytoplasm. This process is energy-dependent, occurs optimally at 37C but not at 4C, and is frequently initiated during adsorption by interaction of viral surface proteins with cellular receptors. For example, internalization of the adenovirus virion is potentiated by the interaction of Arg-Gly-Asp (RGD) peptide sequences in the penton base with secondary host cell receptors, the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. This interaction induces receptor aggregation, which usually triggers a biochemical signal followed by internalization through an endocytic process that involves clathrin-coated pits. Thus, the virus is brought into endosomes in the cytoplasm. *Third*, in many instances uncoating of the virus progresses when the pH of the endosome decreases, thus inducing further biochemical interactions of the viral surface with the endosome membrane, which ultimately lead to release of the viral capsid contents into the cytoplasm.

An important characteristic of viral entry from the targeting standpoint is the level of interdependence of the molecules involved on the steps described above. In effect, the participation of distinct molecules during binding and internalization allows modulating the former without disturbing the second. In this regard, retargeting of adenoviral vectors has been facilitated by the fact that the entry of adenovirus into susceptible cells requires two sets of molecules acting autonomously at two sequential steps, as mentioned above. Thus, targeting maneuvers need only to assure the initial binding step. After anchoring has occurred, which can be determined by binding of the targeted vector with receptors other than CAR, internalization rapidly follows. On the other hand, targeting of retroviruses has been hampered by the participation in viral entry of a single molecular complex of the viral envelope containing multiple domains. As a consequence, most attempts to modify binding have been followed by a parallel dramatic loss of internalization efficiency.

The particular mechanisms whereby viruses enter into human cells are very diverse; so are the targeting strategies and the hurdles encountered for modifying entry of the derived vectors into cells. Based on the aforementioned facts, two main *strategies* have been evaluated for modifying the binding and entry of viral vectors into target cells. These include (i) the use of bifunctional crosslinkers that bind both to the viral vector and to a specific surface receptor in target cells, and (ii) the genetic modification of the viral superficial proteins (**Table 2**).

2. Crosslinker-based (Immunological) Targeting

Targeting by using bifunctional crosslinkers has been reported for adenoviral vectors. We have shown that it is possible to redirect adenoviral infection by employing the Fab fragment of a neutralizing anti-knob monoclonal antibody (mAb) chemically conjugated to a cell-specific ligand. When complexed with preformed adenoviral vector particles, the bispecific conjugate simultaneously ablates endogenous viral tropism and introduces novel tropism, thereby resulting in a truly targeted adenoviral vector. We have employed a number of targeting ligands, including folate, basic fibroblast growth factor, and an antibody directed against the epidermal growth factor receptor. In this manner, it has been demonstrated that tropism-modified adenoviral vectors can infect cells that are refractory to transduction by the native vector; that tropism-modified adenoviral vectors can enhance gene transfer to target cells; and that this enhancement in infection can be translated into a therapeutic benefit in vivo. Others have similarly retargeted adenoviral vectors by means of bispecific antibodies comprising a monoclonal antibody to an epitope engineered in the penton base and a monoclonal antibody to a cell surface receptor. The crosslinker approach to the generation of tropism-modified adenoviral vectors suffers, however, from a number of limitations. In particular, since the targeting ligand is not covalently coupled to the adenovirus particle, there is the potential for the bispecific conjugate to become dissociated from the vector *in vivo*. Production and scale-up of the conjugates may also hamper the widespread clinical application of the strategy. The production of recombinant fusion proteins containing binding domains to the viral vector and to a cell ligand is a more feasible approach, and might advance crosslinker-based strategies to practical implementation.

Two examples are fusion proteins containing two antibody fragments, or diabodies, and the fusion of the soluble adenovirus CAR receptor with a variety of cell ligands.

3. Genetic Targeting

The drawbacks inherent in any strategy to redirect *adenovirus* tropism by complexing the vector particles with bispecific targeting conjugates could be avoided by the direct genetic engineering of the viral capsid proteins to contain cell-targeting ligands. In this regard, the carboxy terminus of the adenovirus fiber protein can be modified to incorporate targeting motifs with specificity for cellular receptors. In an alternative approach, we have also reported that targeting ligands can be incorporated within the so-called HI loop of the fiber knob. Adenoviral vectors which have been engineered to incorporate either a polylysine motif at the carboxy terminus of the fiber or an RGD motif at the carboxy terminus or in the HI loop have demonstrated significantly enhanced infection of cancer cell lines and primary tumor cells which express low levels of the primary adenovirus receptor. Thus, these genetic modifications to the fiber protein have resulted in *expanded* tropism by successfully redirecting adenovirus binding to alternative cellular receptors.

The next challenge will be to employ genetic methods to engineer adenoviral vectors with *restricted* specificity for a single target cell type. In addition to recognizing novel receptors, such vectors should also lack the ability to bind to the native primary adenovirus receptor. This indeed has been accomplished by site-directed mutagenesis of the fiber knob domain to eliminate the cell-binding site. An important consequence of the ablation of native adenovirus tropism is that it will not be possible to propagate these vectors on standard cell lines that express the fiber receptor. However, we have recently developed a novel artificial primary receptor that can be recognized by adenovirus vectors that fail to bind the native fiber receptor. This technology should be useful in the propagation of genetically modified, truly targeted adenoviral vectors.

As said before, and in contrast to adenoviruses, *retroviruses* employ a single envelope glycoprotein to accomplish both binding to the cellular receptor and the subsequent step of membrane fusion. As a consequence, modification of retroviral tropism has proven

problematic, with few reports of modified envelope proteins that retain these two functions of binding and fusion such that efficient delivery can be achieved. A number of molecules, including single-chain antibodies, growth factors and cytokines, have been genetically incorporated into the retroviral envelope glycoprotein, whereupon they confer novel binding specificities into the engineered viral particles. However, some of these surface displayed polypeptides failed to mediate retroviral infection; rather, they proved inhibitory to gene delivery by the modified vectors. In an elegant approach to overcome this obstacle, a protease cleavage site has been incorporated into the design of the retargeted vector. Thus, upon contact with proteases expressed on the surface of the target cell, the inhibitory polypeptide is cleaved from the viral surface, thereby restoring infectivity. To date, tropism-modified retroviral vectors have suffered from significantly lower viral titers than the parental vectors and it is therefore not yet proven possible to employ targeted retroviruses *in vivo*.

4. Target Definition

A key factor in any transductional targeting schema is the availability of appropriate specific molecules on the target cells that can be exploited as specific anchor receptors. To date, proof of principle has been accomplished with a limited range of targeting moieties chosen for their ability to bind to the relatively short list of previously identified cellular receptors. However, a number of groups have described systems that employ high-throughput screening of phage libraries for identifying molecules, including peptides and single-chain antibodies, which bind to specific cell types, both *in vitro* and *in vivo*. Of note, this powerful new technology allows the rapid isolation and screening of potential tumor-specific or tumor vasculature-specific ligands without requiring that the target of the ligand be identified. This approach has already permitted the derivation of cell surface targeted adenoviral vectors for enhanced gene transfer, currently being tested for cancer gene therapy.

D. Transcriptional Targeting

In contrast with surface targeting, which attempts to augment the specificity of gene transfer but, importantly, also seeks to expand vector tropism and to enhance its overall efficiency, transcriptional targeting has been applied mostly for *restricting* the expression of transgenes to target cells. Thus, it has found wide application in the area of molecular chemotherapy, where tumor- or tissue-specific regulatory sequences have been employed to limit the expression of prodrug-converting enzymes specifically to the target cancer cells and dispensable homotypic cells **(Table 3)**. In the same vein, more recently, transcriptional targeting is being used to "untarget" the expression of transgenes from non-target organs that are unduly sensitive to the effect of the transferred gene. For example, it may be desirable to minimize expression of thymidine kinase in the liver, where it induces a limiting toxicity in animal models after intraperitoneal and intrahepatic vector administration. In both cases, by limiting the transgene expression to tissues of a particular type, cycling and angiogenic tissues, or transformed cells, and by diminishing the expression in other normal tissues, a net increase in the therapeutic index may be achieved.

1. Tissue-Targeted Expression

Tumor markers have been used in the clinic extensively for diagnostic purposes. Many were early on considered by vector engineers to represent attractive candidate gene promoters **(Table 3)**. Thus, transcriptionally targeted adenoviral vectors expressing the toxin gene thymidine kinase under the control of the tumor-specific promoter alpha-fetoprotein and prostate specific antigen were employed in molecular chemotherapy approaches to hepatocellular and prostate carcinoma, respectively. The selective expression of the therapeutic gene in the target tumors suggests that transcriptionally targeted adenoviral vectors would be of clinical utility in other diseases. However, it has been reported that certain tumor-specific regulatory elements lose their specificity in the context of an adenoviral vector genome. "Insulator" elements are being engineered in the vector genomes that are able to restrict back the expression of the transgene to the desired tissues. Further limitations come from the prohibitively large size of many regulatory sequences, which exceed the capacity of certain current vectors. However,

novel gene transfer systems with larger capacity are being developed and could be employed to overcome this limitation - these vectors include gutless adenoviral vectors and recombinant herpes virus.

2. Disease-Targeted Expression

A number of genes are overexpressed in cells with high metabolic and proliferating activity. These include not only genes activated in the tumor cells, but also those genes in the stromal cells that, induced by the tumor microenvironment milieu, contribute to sustaining locally tumor progression, such as genes regulating angiogenesis, invasion, and metastasis **(Table 3)**.

3. Externally-Regulated Expression

Certain promoters can be activated regionally by the application of conventional cancer therapies such as radiation, or systemically by drugs such as derivatives of tetracycline and steroids. In both cases, these systems allow to superimpose the timing of expression as an additional level of control in the expression of targeted genes. Further development of the technology seems, however, contingent upon availability of adequate surface and transcriptional targeting systems.

4. Promoter Definition

High-throughput screening of phage display libraries promises to define organ- and tumor-specific ligands. In a technological breakthrough of similar potential, the rapid and precise definition of genes overexpressed and underexpressed in tumor versus normal tissues, and of the corresponding regulatory promoter sequences, is being revolutionized with other high-throughput techniques, including microarrays and serial analysis of gene expression (SAGE). For instance, based on this last method, most transcripts in tumor and normal cells can be quantitated and compared, defining gene expression profiles with a potential value that seems hard to overestimate in the current context.

III. Future Directions

To date, targeted gene therapy has been attempted most frequently by employing either transductional targeting or transcriptional targeting alone. However, it is immediately possible to enhance the overall level of gene transfer specificity by combining the complementary approaches of transductional and transcriptional targeting, each of which might be imperfect or "leaky" by itself. It is also readily possible to enhance the potency and stringency of each targeting strategy by combining several of its modalities, such as deriving "cocktail" approaches that simultaneously exploit the genetic and immunological targeting maneuvers against a variety of tumor cell targets, or by adding dual promoters. It is very possible, however, that the major advances in the field will come from the application of the high-throughput technologies mentioned above, which will allow to define targeting moieties and gene expression regulatory sequences of utmost relevance and precision, at scale. Together with a better understanding of the factors determining vector distribution at the entire organism level, the predictable development of targeted vector systems seems to put the paradigmatic targetable, injectable vectors at our fingertips.

IV. Glossary

Gene therapy: An evolving technique used to treat inherited and acquired diseases. The medical procedure involves replacing, manipulating, or supplementing nonfunctional genes with therapeutic genes.

Targeted virus: A virus that has been modified by genetic or chemical means to direct it towards, or away from, a certain population of cells.

Targeting: A strategy to limit the vector-mediated gene transfer and expression to a certain population of cells. The targeted cells usually share the same histology (for example, prostate) or physiology (highly proliferative, malignant transformation).

Transcriptional targeting: A targeting strategy based on limiting the expression of transferred genes to cells that have a particular regulatory sequence, or promoter, activated. An example is the delivery of toxin genes under the control of the alpha-fetoprotein promoter, which is activated in hepatocarcinoma cells.

Transductional targeting: A targeting strategy based on limiting the binding capacity of the vector system exclusively, or preferentially, to the cell surface of target cells.

Vector: An agent, such as a virus or a small piece of DNA called a plasmid, which carries a modified or foreign gene. When used in gene therapy, a vector delivers the desired gene to a target cell.

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VII. Figure legends

Fig. 1. Targeting of recombinant adenovirus vectors. Adenoviruses have a characteristic morphology consisting of an icosahedral shell composed of 20 equilateral triangular faces and 12 vertices. The protein coat (capsid) consists of hexon subunits and penton subunits at each vertex. A fiber with a knob at the end projects from each penton; this fiber is involved in the process of attachment of the virus particle to the host cell. Genetic modifications into the knob **(A)** endow the fiber with new binding capacities that enable the virus to attach to alternate cellular receptors, thus either augmenting gene transfer efficiency (when the new receptors are more abundant) or making the gene transfer more selective (when the receptors are present preferentially in the target cells **(B)**.

Fig. 2. Broadening of the viral tropism. Cross-linking and genetic methods have been employed to insert the ligand of the CD40 molecule into the viral knob. Such targeted viral vector has an increased capacity to infect otherwise refractory dendritic cells, which express CD40 in their surface, and to thus trigger cell maturation. As a consequence, dendritic cells genetically modified in this manner have an enhanced efficacy for vaccination.

Table 1. Vector Targeting Strategies

Basis Of The Targeting Maneuver	Modality	Example
Physical Delivery	In vitro gene transfer	Infection of T lymphocytes with a retrovirus encoding adenosine deaminase
	Intraarterial infusion	VEGF plasmid infused in coronary arteries
	Intratumoral injection	HLA-B7 DNA-liposome complexes injected into melanoma nodules
	Intraperitoneal infusion	Adenovirus encoding thymidine kinase followed by intravenous ganciclovir
	Intrapleural infusion	Adenovirus encoding thymidine kinase followed by intravenous ganciclovir
Tumor Physiology	Dividing versus non-dividing cells	Treatment of brain tumors using intra-tumoral transduction with a retrovirus encoding the thymidine kinase gene and intravenous ganciclovir
	Tumor hypoxia	(*) Regulation of therapeutic gene with promoter containing a hypoxia responsive element (HRE)
	Tumor vasculature	(*) Angiogenic endothelial cells avidly bind and internalize cationic liposomes and liposome-DNA complexes
	Lower apoptosis threshold	(*) Adenovirus encoding pro-apoptotic Bax induces tumor cell cytotoxicity and radiosensitization without apparent toxicity in normal cells
Cell-Specific Surface Factors (Transduction)	Crosslinker-based targeting	(*) Fab-FGF2 conjugates bind to the adenoviral fiber knob and to FGF2 tumor cell surface receptors, thus allowing in vivo an increased therapeutic ratio
	Genetic targeting	(*) RGD peptide engineered into HI loop of adenovirus knob allows targeting αvß3 and αvß5 integrin receptors
Cell-Specific Regulators of Gene Expression (Transcription)	Tissue-targeted expression	The prostate specific PSA promoter controls the expression of replication-enabling viral vector genes, thus allowing prostate-specific viral replication and oncolysis
	Disease-targeted expression	(*) The endothelin-1 promoter, activated in growing endothelium, can direct the expression of a therapeutic gene to areas of angiogenesis
	Externally regulated expression	(*) A radio-inducible suicide gene (EGR-tk) can be constructed by cloning the early growth response (Egr)-1 promoter upstream of the herpes simplex virus thymidine kinase (HSV-tk) gene

(*) Animal models only.

PSA: prostate specific antigen; RGD: tripeptide Arg-Gly-Asp

Table 2. Transductional (Surface) Targeting

Targeting strategy	Modality	Example (vector – applications)
Crosslinker-based Targeting	Immunological	Ad – Fab portion of anti-adenoviral knob antibody chemically linked to ligand, for instance Fab-folate conjugates for targeting into ovarian cancer cells
		Ad – Bispecific single-chain antibodies that bind to Ad knob and to EGF receptor
Genetic Targeting	Modification of viral coat proteins	Retro – insertion of erythropoietin in env protein
		Ad – insertion of RGD ligand in HI loop and in carboxy-terminus
		HSV – insertion of erythropoietin in envelop protein
		AAV – CD34 and bispecific Fab2 antibody
		Sindbis and HCG
	Ablation of binding	Ad – mutations of knob and penton base ablate binding into natural receptors, CAR and integrins
	Pseudotyping	Retro – replace the coat proteins of the vector with the coat proteins of another virus endowed with desired tropism
	Hybrids	Ad – substitution of the fiber with an artificial structure that possesses alternate targeting ligands
		Ad – construction of vectors with multiple fibers of distinct binding properties
Combination of crosslinker and genetic targeting	Biotin-Avidin based	Several vectors – Biotinylation of viral coat or envelope proteins, which can be facilitated by genetically engineering biotin acceptor tags, allows combination with targeting moieties complexed with avidin
		Retro – display Ig binding domain on vector as genetic fusion with coat protein; use mAb to crosslink vector with targeted cell

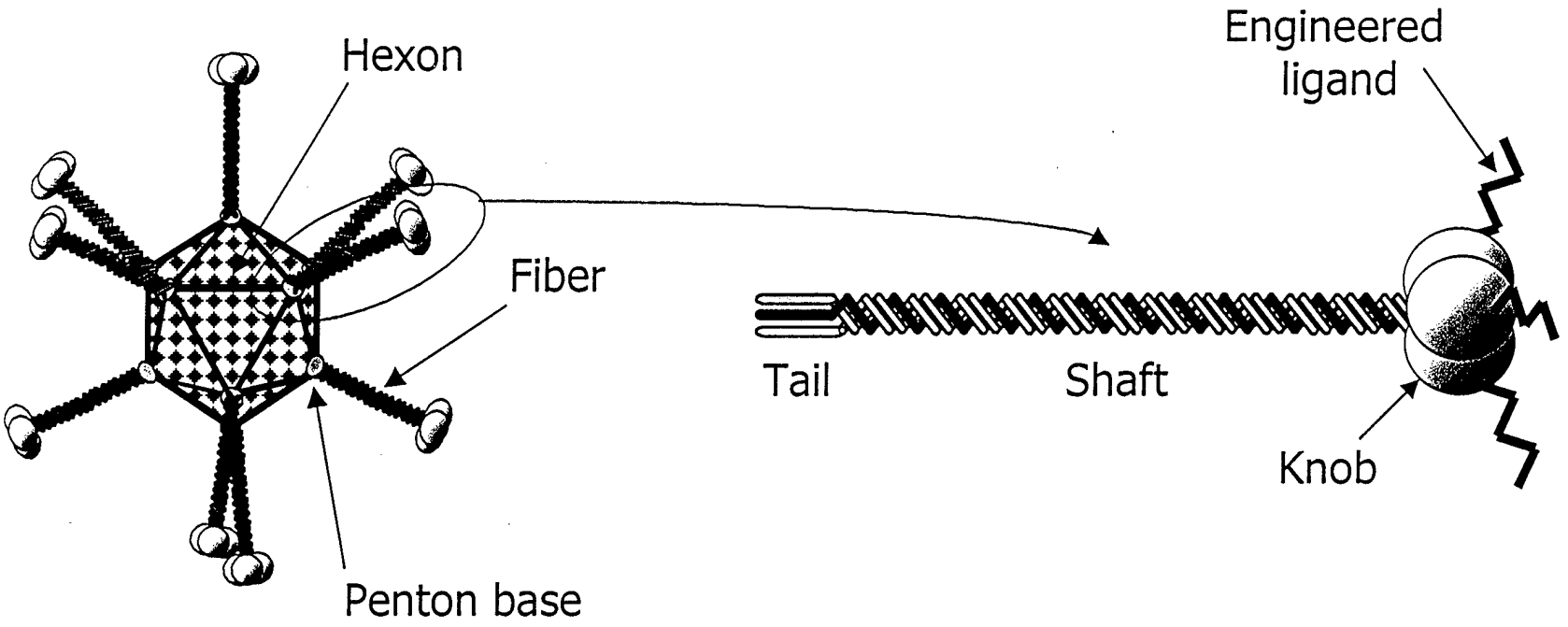
Ad: adenovirus; CAR: coxsackie and adenovirus receptor; Ig: immunoglobulin; retro: retrovirus

Table 3. Transcriptional (Promoter) Targeting

Promoters	Organ or condition of expression
Tissue-specific promoters	
α-fetoprotein	Hepatoma
ß-casein	Breast
Calcineurin α	Neuroblastoma, glioblastoma
Carcinoembryonic antigen	Colon, lung
DF3/MUC	Breast
erbB-2	Breast, pancreas, gastric
HSV-LAT	Neuroblastoma, glioblastoma
Ig heavy and light chain	B-cell leukemia and lymphoma
Myelin basic protein	Glioblastoma
Osteocalcin	Osteosarcoma
Prostate specific antigen	Prostate
sis	Osteosarcoma or chondrosarcoma
Secretory leukoprotease inhibitor	Colon, lung, breast, bladder, oropharyngeal, ovarian, endometrial
Synapsin 1	Neuroblastoma, glioblastoma
Tyrosinase	Melanoma
Tyrosinase-related protein 1	Melanoma
Disease-associated promoters	
COX-2	Tumor cells
Cyclin A	Proliferation
Endoglin	Angiogenesis
Endothelin-1 promoter	Ischemia
E2F-1	Proliferation
E-selectin	Angiogenesis
Glucose response protein 78	Acidic, anoxic, glucose-starved tumor tissue
Hypoxia response element	Hypoxia
L-plastin	Tumor cells
Midkine	Tumor cells
Myc-Max responsive element	Lung
Phosphoglycerate kinase promoter	Hypoxia

Vascular endothelial growth factor	Hypoxia and other stimuli of angiogenesis
Inducible systems	
Early growth response gene -1	Radiation
Heat-shock protein 70	Hyperthermia
Multiple drug resistance	Chemotherapy
Tet	Tetracycline, doxycycline

DF3/MUC: high molecular weight mucin-like glycoprotein; HSV-LAT: herpes simples virus latency-associated transcript; Ig: immunoglobulin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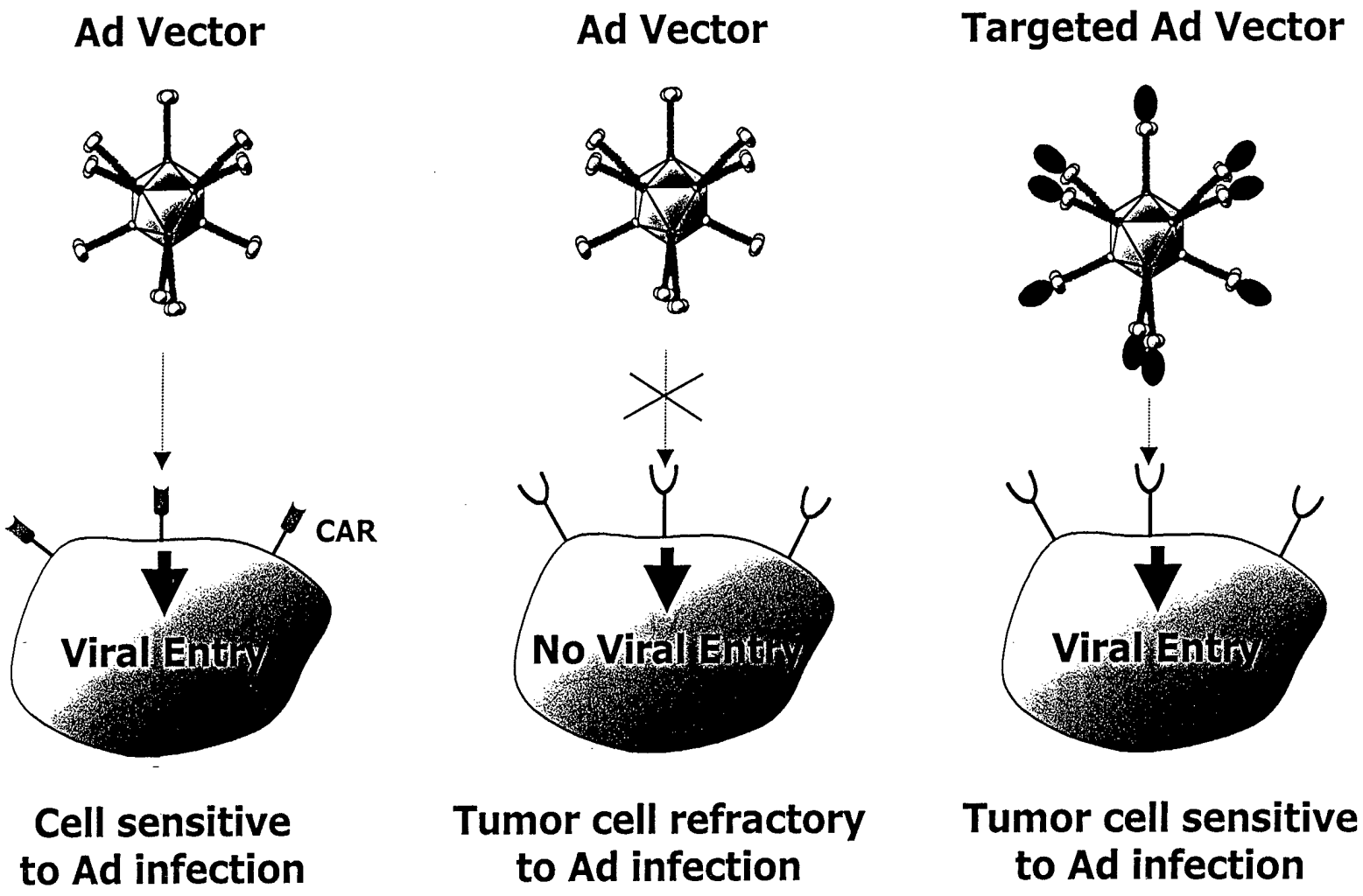
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Tumor cell refractory to Ad infection
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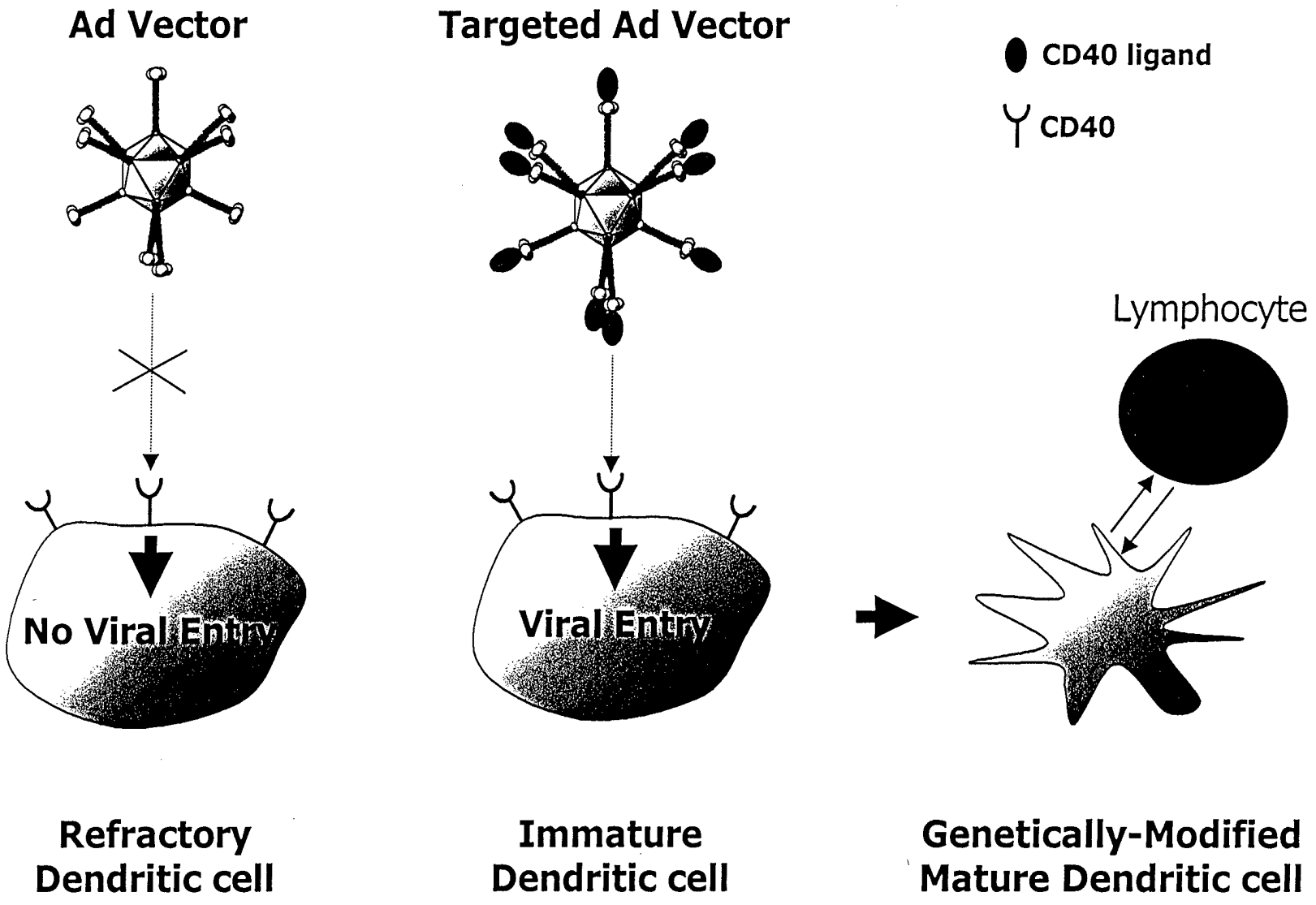
Tumor Cell Receptor
Targeting Moiety


Ad Vector
Targeted Ad Vector
CD40 ligand
CD40
Lymphocyte
No Viral Entry
Viral Entry
Refractory Dendritic cell
Immature Dendritic cell
Genetically-Modified Mature Dendritic cell

Human papillomavirus E6E7-mediated adenovirus oncolysis: selectivity of mutant adenovirus replication ascertained in organotypic cultures of human keratinocytes.

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Abstract

The causal link between human papillomavirus (HPV) infections and anogenital squamous cell carcinomas has been well established. HPV-associated cancers consistently express papillomavirus early 6 and 7 oncoproteins known to cause cell immortalization and transformation. Here we propose a viral therapeutic approach for HPV-associated cancer based on HPV E6E7-dependent cell killing by an adenovirus E1A mutant. We have studied the replication of this mutant in an organotypic model of stratified squamous epithelia expressing HPV18 E6E7 oncoproteins. Our results demonstrate that this mutant can selectively replicate in and induce lysis of keratinocytes that express the viral oncoproteins critical to HPV-induced carcinogenesis.

Numerous reports have established the causal association of human papillomavirus (HPV) infections with squamous carcinomas and adenocarcinomas of the anogenital tract [1,2]. Over 95% of anogenital tumors contain HPV sequences that belong to the oncogenic group (HPV16, 18, and related types) [2], and consistently express papillomavirus early 6 and 7 oncoproteins. E6 and E7 oncoproteins interact with the master cell cycle regulatory proteins, p53 and Rb, respectively, and are able to immortalize primary keratinocytes *in vitro* and cause cell transformation in cooperation with other oncoproteins like Ras [3]. Furthermore, continuous expression of E6 and E7 oncoproteins is necessary for the maintenance of the transformed phenotype. These properties have made E6 and E7 targets of various experimental therapeutics including vaccination-, RNA antisense-, and ribozyme-based approaches [4,5], encountering only limited success.

Replication-competent adenoviruses (Ad) as oncolytic agents are emerging as new therapeutic anti-cancer drugs (for a review, see [6]). New efforts in this growing area have concentrated on achieving tumor-selective adenovirus replication. In this regard, two major approaches have been developed to restrict adenovirus replication to tumor cells, namely

controlling adenovirus early gene expression by a tumor-specific promoter [7] and introducing viral genomic deletions that affect viral protein functions dispensable in cancer cells [8,9]. Despite the conceptual validity of these approaches, demonstrating replication selectivity has proven difficult due, in part, to the incomplete knowledge of all viral protein functions and to the lack of appropriate models to study adenovirus replication in a physiological setting. The functional similarities between certain papillomavirus and adenovirus proteins may provide a window of opportunity to fulfill the requirements of replication selectivity.

Both papillomaviruses and adenoviruses have evolved a similar mechanism to usurp the cell cycle regulation in order to facilitate viral DNA replication [10,11]. The early adenoviral region 1A (E1A) is the first transcription unit to be expressed upon infection, and is required for cell cycle mobilization and *trans*-activation of other virus early promoters, leading to viral DNA replication. The E1A polypeptides encoded by the 12S and 13S mRNAs share sequence and functional similarities with the HPV E7 protein in the conserved regions (CR) 1 and 2. E7 and E1A bind and inactivate pRb and related pocket proteins (p107 and p130) through these domains. The *trans*-activation function of the E1A 12S product lies in CR1 and CR2 and is directed at the release of the E2F transcription factor from pRB complexes, resulting in S phase entry as well as adenovirus E2 gene transcription. Interestingly, this E2F-mediated *trans*-activation of the E2 promoter is shared by the HPV16 E7 protein[12]. The E1A 13S product contains an extra domain, the CR3, which is necessary for activating E1b, E2, E3, and E4 viral early promoters. However, efficient activation of viral early promoters by the 13S product also requires the CR1 domain as E1A mutants containing large CR1 deletions, affecting binding to both pRb and p300, activate viral early promoters poorly [13]. In this context, the HPV16 E7 protein has been shown to restore the transcriptional activity of CR1 deletion adenovirus mutants[14]. We present here a novel approach to restrict adenovirus replication to HPV-associated cancer cells based on functional complementation of an Ad E1A deletion mutant by HPV E6E7 oncoproteins.

Results

Given the functional similarity between the HPV E7 and Ad E1A CR1/CR2 domains, we hypothesized that CR1 and CR2 are both dispensable for adenovirus replication in the presence of HPV E7. Therefore, we generated an adenovirus containing two deletions (amino acids 27-80 and 121-129) that affect the CR1 and CR2 domains (Figure 1a). This mutant, named CB016, expresses a truncated set of E1A products unable to bind p300, pRb and p107, in contrast to the wild type E1A protein (Figure 1b).

We sought a model to study CB016 differential replication in HPV-positive and HPV-negative cells. Because of the strict species-specificity of human adenovirus, the use of animal models is obviated. We thus considered employing cultures of human HPV+ cell lines and of primary human keratinocytes. However, submerged cultures may have different properties from epithelial tissues *in vivo* [15] and further, the ability of adenovirus to replicate varies upon the epithelial tissue origin and differentiation stage [16]. For these reasons, we chose to perform this study in human organotypic cultures of primary neonatal keratinocytes (hereafter referred as raft cultures) which closely reproduce the *in vivo* keratinocyte differentiation and stratification programs [17]. To recapitulate the expression of HPV E6E7 oncogenes in this model, we employed a recombinant retrovirus carrying an HPV18 E6E7 expression cassette under the control of the native HPV enhancer and E6 promoter located in the upstream regulatory region (URR). This promoter is repressed in the basal/parabasal strata but is up-regulated upon squamous differentiation into spinous and granular cells [15]. Thus, E6E7 expression occurs in differentiated cells as opposed to basal cells such that E7 re-establishes S phase in a subset of post-mitotic keratinocytes [18]. By using this model, replication of various adenoviruses can be examined in normal proliferating basal/parabasal cells, in post-mitotic, differentiated cells, as well as in differentiated cells expressing HPV18 E6 and E7.

Because complementation of adenovirus E1A and E1b deletion mutants in HPV-positive cells has been previously observed [19], we studied the replication of CB016 and other E1A deletion mutants, namely Ad dl312 [20] (an entire E1A deletion mutant) and Adßgal [21] (an

E1A/E1B-deleted adenovirus containing a ßgal expression cassette in the deleted region). Human foreskin keratinocytes were transduced with an HPV18 E6E7 retroviral vector, and then infected with various adenoviruses at an MOI of 1. The infected cells were trypsinized, transferred onto a dermal equivalent, and allowed to differentiate at the air-medium interface for 9 days. To monitor E7-mediated reactivation of cell cycle, cultures were labeled with BrDU 12 h prior to harvesting.

Figure 2 shows the histology of control raft cultures and cultures infected with retroviruses, adenoviruses or both. Adwt300 infection caused a widespread cytopathic effect in all the cultures with or without HPV E6E7. The epithelium was disorganized and condensed cell nuclei were abundant. Nevertheless, the presence of several cell layers indicates that the replication of Adwt300 in the early phases of raft culture development does not completely prevent epithelium stratification, suggesting that basal cell killing must have occurred at a later stage.

Adßgal infection did not produce any cytopathic effect in the infected raft cultures, regardless of the expression of E6E7. We did not detect Adßgal replication in the normal or E6E7 raft cultures, even by *in situ* hybridization of the adenovirus genome (data not shown). To substantiate Adßgal infection, we monitored the expression of the ß-galactosidase transgene by X-gal staining. In uninfected raft cultures, no ß-galactosidase activity was detected (not shown). In infected cultures, ß-galactosidase activity was detected only in the enucleated outer squames (Figure 2). We suggest that some cells quickly migrated up the epithelium with no or limited rounds of cell division. Because of the relatively high levels of ß-galactosidase expression prior to stratification, the residual signals remain detectable in the squame. In contrast, if the infected cells had divided multiple times, the input adenovirus genomes would have been diluted in the absence of viral DNA replication such that ß-galactosidase activities became too low to be detected. It is also likely that, unlike the URR-E6 promoter, the CMV promoter driving the transgene expression has little or no activity in differentiated keratinocytes. Similar to Adßgal, Ad dl312 infection revealed no cytopathic effect in normal or E6E7-expressing keratinocytes. Taken together, these results indicate that,

at the MOI used in these experiments, adenoviruses deleted of the entire E1A gene cannot be complemented for replication in E6E7-expressing raft cultures.

In contrast to Adwt300, Ad dl312, and Adßgal, CB016 showed a distinct pattern of replication in the normal and E6E7-transduced raft cultures. In normal cultures, CB016 produced no cytopathic effect in the main central section (Figure 2) with only localized cytopathic effect in the margin of the cultures (not shown). However, in E6E7-transduced cultures, cytotoxicity was confined to the differentiated strata normally comprising spinous and granular cells, where widespread necrotic lesions were evident. The basal/parabasal strata were spared from this cytopathic effect, in agreement with the spatial distribution of E6E7 expression.

To ascertain that the histopathology observed in CB016-infected raft cultures was attributed to adenovirus replication and progeny production, we performed immunofluorescence staining to detect BrDU, Ad E1A protein, and Ad late protein hexon. As shown in Figure 3, in normal cultures incorporation of BrDU was confined exclusively to the basal/parabasal proliferating cells. In normal CB016-infected cultures, E1A and hexon expression was scarce, with the exception of the margin of the cultures (not shown). In uninfected E6E7-transduced cultures, incorporation of BrDU was detected in a subset of both basal and suprabasal cells, as described previously [18]. In CB016-infected E6E7-transduced raft cultures, Ad E1A and hexon proteins were localized within the suprabasal necrotic areas previously identified by hematoxylin/eosin staining. Furthermore, DNA *in situ* hybridization of the adenovirus genome confirmed the presence of adenovirus DNA in the nuclei of the cells staining positive for hexon (not shown). However, only cells in the basal/parabasal strata incorporated BrDU. We conclude that the adenovirus had specifically replicated in and lysed the E6E7-expressing differentiated suprabasal cells prior to exposure to BrDU. Taken together, these results indicate that CB016 replication is complemented in HPV E6E7 expressing keratinocytes.

We also analyzed the effect of HPV18 E7 expression alone [18] in the replication of CB016. Primary human keratinocytes transduced with a retrovirus expressing E7 under the E6

natural promoter in the URR, and infected with CB016, also exhibited signs of adenovirus induced lysis in the upper strata (Figure 2 and 3). However, this effect was less pronounced than in the E6E7-transduced cultures. Not only the basal/parabasal but also the lower spinous cells were spared. Whether this reduced cytopathic effect is due to lower level of E7 expression remains to be determined. However, it has been a consistent observation that a higher percentage of the differentiated cells re-enter S phase in the E6E7-transduced cultures than those transduced by E7 alone (Noya, Chien, Broker and Chow, unpublished and also see Figure 3). Alternatively, a possible role of E6 in complementation of CB016 replication cannot be ruled out since E6 has been recently shown to bind the transcriptional coactivator p300 [22], which is also a target of Ad E1A.

Finally, we examined the temporal replication of CB016 in the raft cultures. For this purpose, three CB016-infected normal or E6E7-transduced culture replicas were harvested at days 8, 11, and 14 post-infection. By day 8, early signs of CB016 replication in E6E7-transduced cultures appeared in scattered cells within the upper strata (Figure 4). These cells showed condensed nuclei and vacuolated cytoplasm. On day 11, a wide necrotic suprabasal area was evident and remained unchanged through day 14. In normal cultures (Figure 4), the earliest time point revealed a properly stratified and differentiated epithelium. By day 11, and subsequently by day 14, the infected epithelia became increasingly thinner in width. However, this effect was not accompanied by the necrotic lesions observed in the E6E7-transduced cultures.

To monitor the expression of early and late adenovirus proteins over time, an immunofluorescence analysis was performed (Figure 5). This analysis revealed the presence of scattered E1A-positive cells in day-8 normal raft cultures, with little or no concomitant hexon expression. In contrast, day-8 E6E7-transduced raft cultures showed large areas of E1A expression in the upper strata. Colocalization of BrDU and E1A was evident in the majority of E1A-positive cells, indicating that the virus DNA was being actively replicated at the time of BrDU labeling. Hexon-positive cells were concomitantly detected in the same area. On day 14, normal raft cultures showed the presence of basal proliferating cells mixed with

E1A/hexon-positive cells. In E6E7-transduced raft cultures, E1A and BrDU-positive cells were confined to the basal-lower squamous strata. Hexon signal, however, was mostly concentrated in the necrotic lesion developed within the upper strata. Taken together, these results indicate that while CB016 replication is enhanced in E6E7-expressing cells, it is not completely abrogated in normal keratinocytes, resulting in a delayed and reduced cytopathic effect.

Discussion

Adenovirus-mediated oncolysis is a rapidly growing field with the potential for conceptualization of new agents [6]). In addition to the two previously described strategies to achieve tumor-specific adenovirus replication, we have proposed here a new approach based on complementation of Ad mutant by HPV oncoproteins. By employing organotypic cultures of human keratinocytes, we have shown that a CR1/CR2-deleted E1A Ad mutant can efficiently replicate in and lyse HPV18 E6E7-expressing keratinocytes. However, in the conditions used in these studies, CB016 replication is not completely abrogated in normal keratinocytes resulting in a delayed toxicity. A possible virus dose effect warrants further investigation. Since CB016 still carries the transcriptional activation domain of E1A, lower doses may result in reduced background trancription. Alternatively, we cannot rule out the presence of an E1A-like activity capable of *trans*-complementing the replication of CB016 in keratinocytes. Several studies have shown that Ad E1A deletion mutants can be transcriptionally activated by cellular proteins with E1A-like functions [23,24]. In this regard, we have observed a cytopathic effect in normal keratinocyte raft cultures infected with E1A-deleted Ads (Ad dl312 and Adßgal) at high MOIs (10 to 100 pfu/cell) (not shown). Interestingly, an E1A-like activity induced by IL-6 has been shown to regulate E1A-responsive promoters in the absence of E1A [25]. Such activities may be responsible for the localized cytopathic effect observed in marginal areas of the normal infected cultures. The

margins are generally populated with keratinocytes attempting to expand outward rather than upward. We speculate that the expression of particular cytokines could be different at the margins versus the internal region, influencing virus replication at different rates.

An imminent therapeutic application for this mutant would be as an oncolytic agent in HPV-associated cancer. In addition, given the role of HPV E6 and E7 proteins in the pathogenesis of both high and low risk HPVs, the panel of therapeutic applications for this type of mutant virus may span from its employment in the treatment of HPV-associated malignant and pre-malignant lesions (i.e. dysplasias to carcinomas) to benign lesions induced by non-oncogenic HPV types. Further studies on the complementation by other HPV types or for other Ad mutants are warranted.

In summary, complementation of adenovirus replication by viral oncoproteins responsible for HPV-induced carcinogenesis in combination with the study of adenovirus replication in a human organotypic model resembling the *in vivo* context are novel incorporations to the field of replicative adenoviruses with broad therapeutic implications.

Methods

Adenoviruses. Adwt300, Ad dl312, and Adßgal were grown in 293 cells and purified by CsCl as previously described [26]. Virus preparations were titered by plaque assay in 293 cells. CB016 virus was generated as follows. A derivative of plasmid pXC1 (containing the left 5766 bp of Ad5) (Microbix, Hamilton, Canada) was made by religating after digestion with XbaI and NdeI. Then, a deletion spanning amino acids 27 to 80 of E1A was introduced by using an oligonucleotide (5' CAG CTG ATC GA GAG CTC ACT TTT CCG CCG 3') flanking the deleted sequence as instructed in the Transformer® site-directed mutagenesis kit (Clontech, Palo Alto, CA). A fragment EcoRI-BspEI was cut out from the deleted plasmid and cloned in the same sites in pXC1-Δ24 shuttle vector containing a deletion from amino acids 121 to 129 of E1A [9]. The resulting plasmid (CB016) was cotransfected with pBHG10 (Microbix, Hamilton, Canada) in 293 cells. Plaques were expanded and viral DNA extracted by a

spermine-based method and sequenced. A plaque showing the correct E1A sequence was further expanded for CsCl banding. Virus stocks were titered by plaque assay in 293 cells.

For keratinocyte infection and in order to calculate adenovirus dose properly, a duplicate well was trypsinized and counted, and well replicas were exposed to adenovirus at a multiplicity of infection of 1, for 4 h. Upon transferring onto dermal equivalents, a small cell suspension aliquot was placed in an 8-well chamber slide for adenovirus E1A immunostaining (Adwt300 and CB016) or X-gal staining (Adßgal) 24 h later. These assays revealed that over 90% of keratinocytes had been infected.

Immunoprecipitation and Western blot. SiHa cells grown in 10-cm culture dishes were infected with Adwt300 or CB016 at an MOI of 20. Fifteen hours after infection, cells were scraped off the plates and cell pellets lysed in 50mM Tris pH 8.0, 5 mM EDTA, 0.1% Triton X-100, 250 mM NaCl, for 30 min on ice. Lysates were cleared by centrifugation and then immunoprecipitated with 0.5 µg anti-E1A mouse monoclonal antibody M73 (Oncogene Research, Boston, MA), plus 20 µl Protein A/G agarose (Santa Cruz Biotech, Santa Cruz, CA), for 3 h at 4°C. Immunoprecipitates were washed 3 times in cold lysis buffer and resuspended in electrophoresis sample buffer. Samples were split and electrophoresed in 7.5% or 12% acrylamide gels, transferred to PVDF membranes, and immunoblotted with either anti-E1A M73 monoclonal antibody (Oncogene Research, Boston, MA), mouse anti-Rb monoclonal antibody, rabbit anti-p107 polyclonal antibody, or rabbit anti-p300 polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA) in TBS-Tween 20 0.2%, at a concentration of 1 µg/ml, 1 h at RT. Membranes were washed three times in TBS-Tween 20 0.5% and incubated with the appropriate peroxidase-conjugated anti-mouse Ig or anti-rabbit Ig antibodies (Amersham Pharmacia, Piscataway, NJ) at a 1:200 dilution in TBS-Tween 0.2%, 1 h at RT. Membranes were washed and developed by chemoluminiscence (ECL, Amersham).

Recombinant retroviruses and raft cultures. The structure and generation of the retroviruses used in this study have been described elsewhere [15]. Organotypic raft cultures of

neonatal foreskin keratinocytes were essentially prepared as described [15]. In the present study, keratinocytes were infected with adenovirus prior to transferring the cells onto dermal equivalents. Raft cultures were allowed to differentiate for the time indicated in the text. BrDU was added to a final concentration of 50 μg/ml for 12 h prior to harvesting. Cultures were fixed in formalin for 3 h and paraffin-embedded. Four-μM sections were stained with hematoxylin/eosin following standard procedures. To detect ß-galactosidase activity, cultures were fixed in 2% formaldehyde, 0.2% glutaraldehyde for 1 h and then incubated with X-gal (1 mg/ml) in PBS containing 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM $MgCl_2$, O/N at 37°C, before being paraffin-embedded.

Immunofluorescence. For antigen retrieval, sections were deparaffinized, rehydrated and treated with 10 mM citrate buffer pH 6.0 at 95°C for 10'. For double detection of BrDU and E1A, antibody reactivity to E1A was first revealed with the anti-E1A mouse monoclonal antibody M73 (Oncogene) at a final concentration of 2 μg/ml followed by Alexa 594-coupled goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR) at a 1:200 dilution. A final incubation was performed with FITC-labeled anti-BrDU monoclonal antibody (1 μg/ml) (Boehringer Manheim, Indianapolis, IN). For hexon staining, sections were incubated with goat polyclonal anti-Ad2 hexon antibody (Chemicon, Temecula, CA), at a 1:300 dilution, followed by incubation with Alexa 588-conjugated donkey-anti goat secondary antibody (Molecular Probes) at a 1:200 dilution. All sections were mounted with Gel/Mount (Biomeda). Photomicrographs in Figures 3 and 5 were captured with either a Texas Red or FITC filter in an Olympus IX70 inverted fluorescence microscope. Individual images were merged by means of the Adobe Photoshop® 5.5 application program.

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Figure legends.

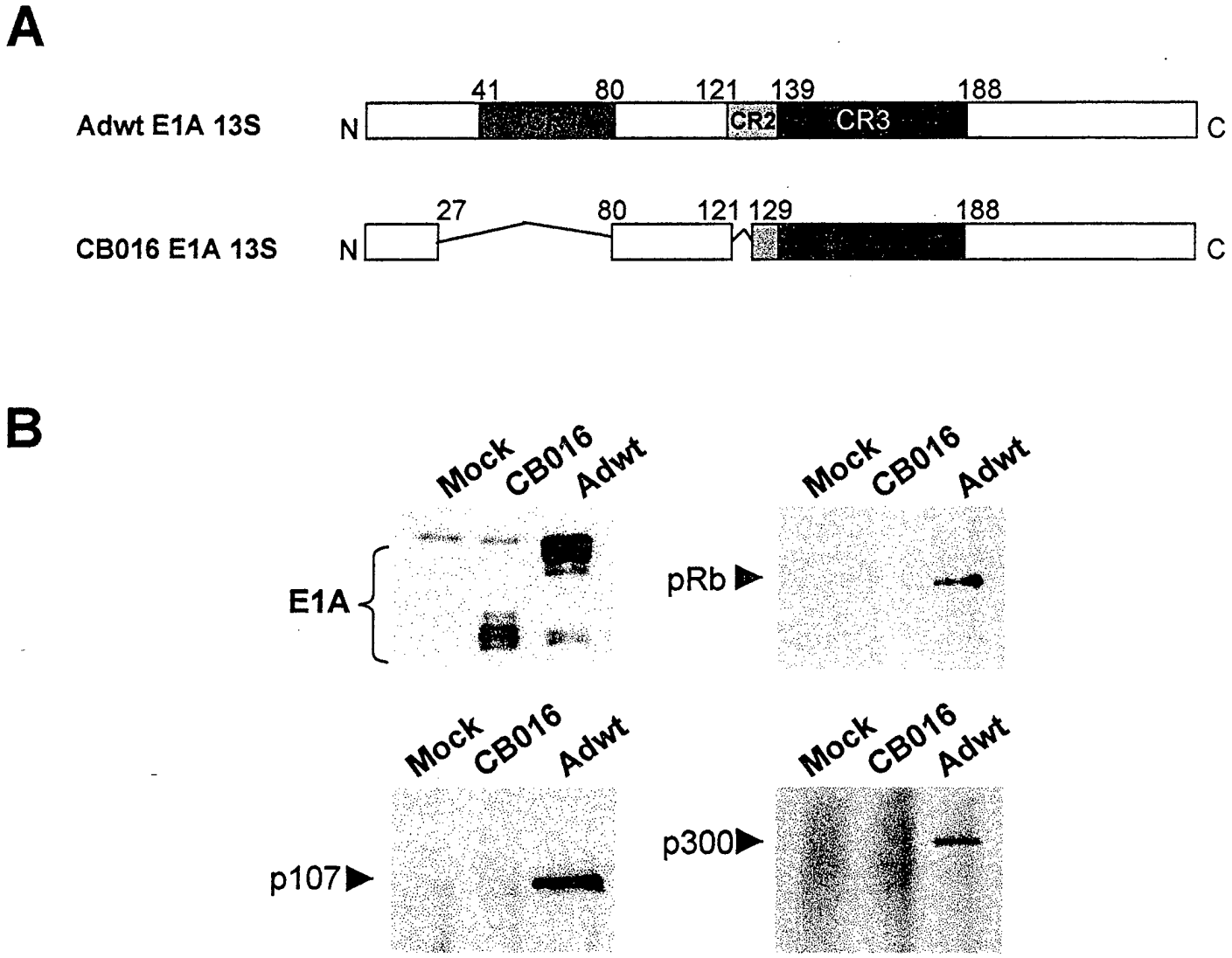
Figure 1. a) Structure of CB016 adenovirus E1A region showing the deletions introduced in the CR1 and CR2 domains of E1A. b) Immunoprecipitation of E1A and E1A-bound cellular proteins. SiHa cells were mock-infected or infected with CB016 or Adwt, and E1A was immunoprecipitated in non-denaturing conditions. E1A immunoblotting reveals a truncated set of E1A protein species for CB016 as opposed to Adwt; co-precipitation of pRb, p300, and p107, by CB016 E1A is abrogated.

Figure 2. Effects of adenovirus infection in raft cultures of normal, HPV18 E6E7-, and HPV18 E7-expressing keratinocytes. Hematoxylin/eosin (H/E) staining of day-9 raft culture sections. Keratinocytes were infected with the indicated adenoviruses at an MOI of 1, prior to being transferred to dermal equivalents (see methods), and allowed to stratify for 9 days. Adßgal-infected cultures were stained with X-gal prior to H/E staining. Blue X-gal staining is indicated by arrowheads.

Figure 3. Immunofluorescence analysis of adenovirus early and late proteins in day-9 CB016-infected normal, HPV18 E6E7-, and HPV18 E7-transduced raft cultures (same experiment as in Figure 2). BrDU and hexon are revealed by green fluorescence, while E1A is revealed by red fluorescence. BrDU and E1A were detected by double immunofluorescence on the same section. Hexon immunofluorescence corresponds to the same field of a serial section. Magnification: 200X

Figure 4. Time-course of adenovirus infection in CB016-infected normal and E6E7-transduced raft cultures. H/E staining of day 8, day 11, and day 14 sections. Arrowheads indicate cells showing early cytopathic effect in E6E7 raft cultures. Magnification: 200X

Figure 5. Time-course of adenovirus infection in CB016-infected normal and E6E7-transduced raft cultures (same experiment as in Figure 4). Immunofluorescence staining for BrDU, E1A and hexon performed as for Figure 3. Co-localization of BrDU and E1A in the same cell yields a yellow fluorescence signal. Magnification: 200X.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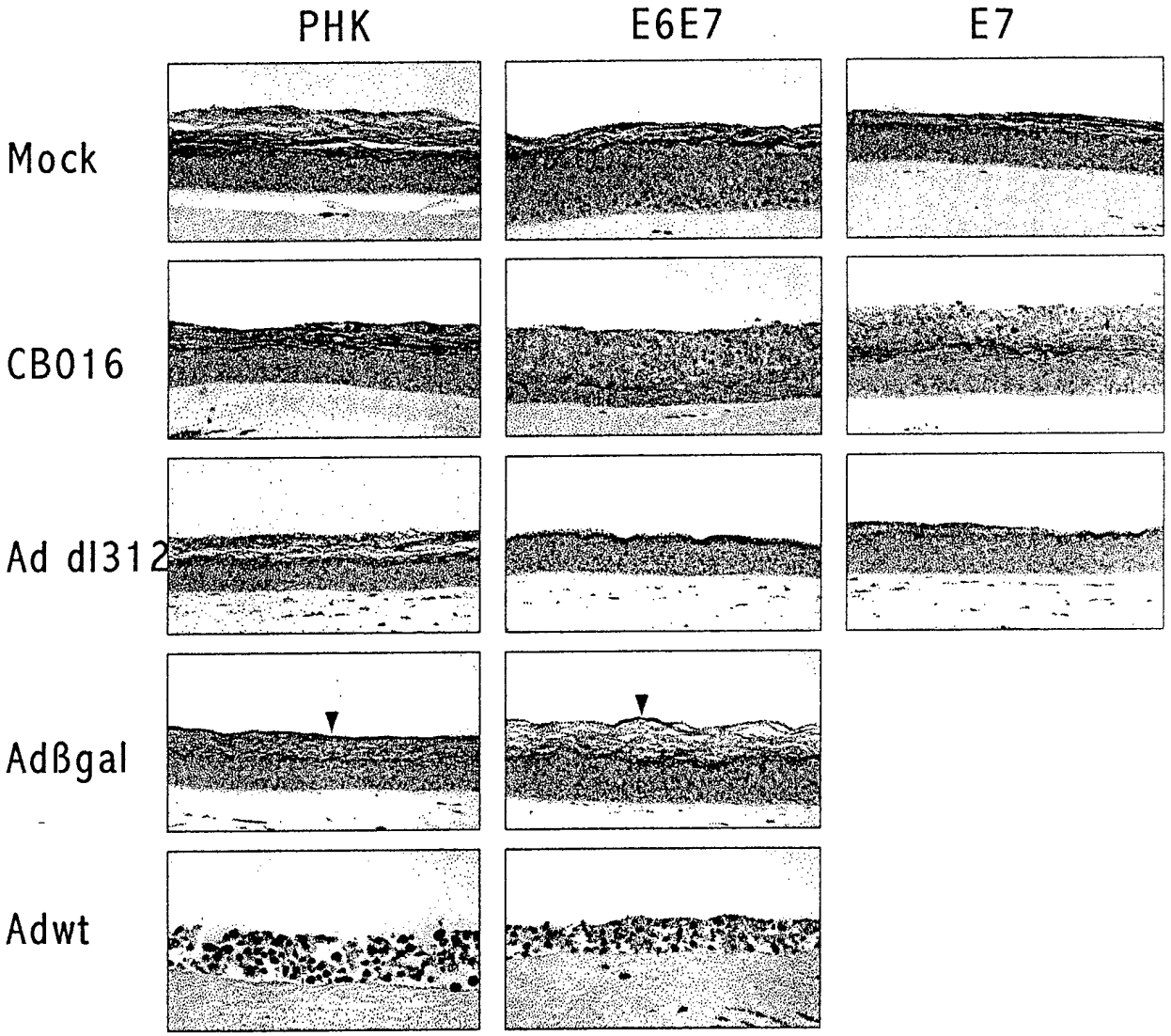
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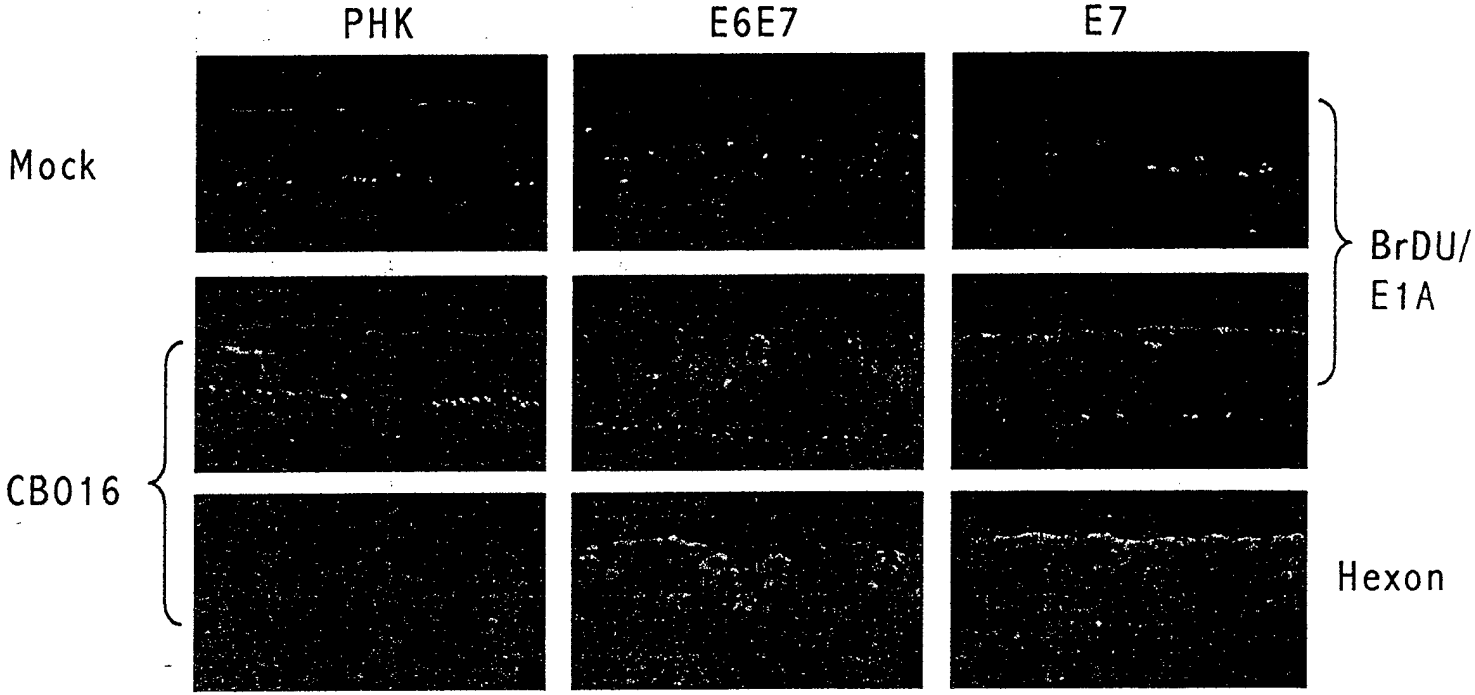
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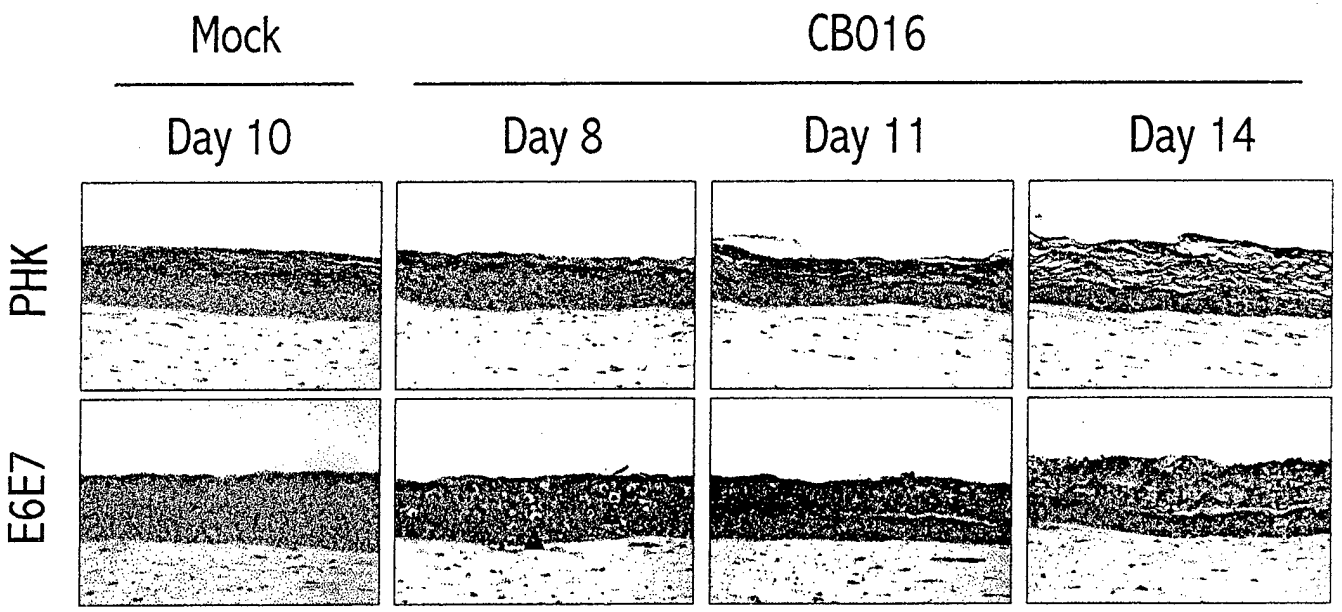
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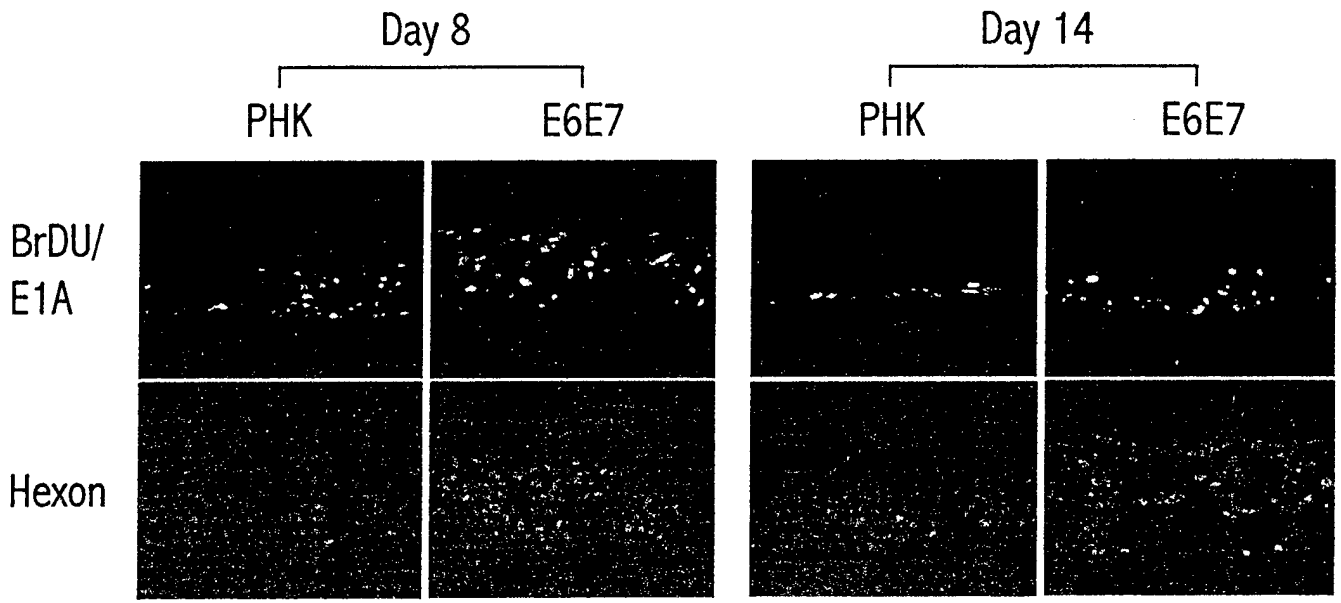
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TRANSCRIPTIONAL TARGETING

FOR OVARIAN CANCER GENE THERAPY

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Abstract

Ovarian carcinoma is a leading cause of cancer death in women. Though advances in conventional therapies have been achieved, long-term survival rates for most patients diagnosed with ovarian cancer are still low. Therefore, novel molecular therapeutic strategies such as gene therapy are being intensively pursued. Such approaches are based on the enormous progress which has been achieved in the elucidation of the molecular foundations of ovarian cancer. In this regard transcriptional control elements (promoters) of genes frequently upregulated or specifically expressed in tumors can be applied in a heterologous context to drive expression of therapeutic genes in targeted gene therapy strategies. This review will discuss transcriptional targeting strategies in ovarian cancer gene therapy and give an overview of tumor-specific promoters (TSPs) which have been applied for this purpose.

Key Words

Transcriptional Targeting, Tumor-specific Promoters, Ovarian Cancer, Gene Therapy

Introduction

Ovarian cancer afflicts over 25,000 women annually in the United States. Due to the lack of effective prevention and screening modalities, the majority of patients who are diagnosed with epithelial ovarian cancer present with advanced stage disease [1]. Advances in surgical technique and chemotherapy have resulted in response rates that exceed 70%, however, most patients with advanced stage ovarian cancer will recur [2]. As such, the five-year survival rate for patients diagnosed with advanced stage ovarian cancer is approximately 15-30% and most patients will ultimately succumb to their disease. These ominous statistics justify the search for effective new therapies, such as gene therapy, for patients afflicted with ovarian cancer.

There is a rational basis to pursue gene therapy as a novel paradigm for ovarian cancer. In this regard, it has become apparent that transformation of normal tissue leading to the emergence of a malignant phenotype results from the accumulation of a series of acquired genetic lesions that enhance tumor cell survival and proliferation; induce invasion, neovascularization and metastasis; and inhibit immunologic surveillance and apoptosis. These processes are becoming increasingly elucidated in the context of ovarian cancer and offer opportunities to be manipulated by genetic means for therapeutic gains. The majority of clinical cancer gene therapy trials to date have depended upon compartment cancer models due to current limitations in gene delivery systems. The ability to concentrate vector in the abdominal "container", the ease of access to the abdominal cavity, and the usual confinement of ovarian cancer to the abdominal cavity

have made ovarian cancer an ideal model to investigate a variety of gene therapy approaches.

Effector Strategies in Cancer Gene Therapy

Current approaches to cancer gene therapy can be divided into four broad categories: a) mutation compensation; b) molecular chemotherapy; c) genetic immunopotentiation; and d) genetic modulation of resistance/sensitivity. Strategies to achieve mutation compensation are designed to rectify the molecular lesions in the cancer cell responsible for malignant transformation. This can involve replacement of a defective tumor suppressor gene (such as BRCA1 or p53) [3,4], ablation of a dominant oncogene (such as erbB-2) [5], or manipulation of genes (such as bcl-2 or bax) that control apoptosis [6,7]. Molecular chemotherapy methods are designed to eradicate tumor cells by the selective delivery or expression of a gene encoding a prodrug converting enzyme ("suicide gene") and subsequent application of the corresponding prodrug, e.g. herpes simplex virus thymidine kinase (HSV-tk) / GCV, or *E. coli* cytosine deaminase (CD) / 5-FC [8,9]. Due to a bystander effect, i.e. killing of non-transduced neighboring cells, quantitative transduction is not necessary in the latter approach. Genetic immunopotentiation is defined as the introduction of genetic modifications into host or cancer cells in order to augment the immunologically-mediated destruction of tumor cells [10]. Lastly, investigators have utilized a variety of gene therapy strategies to alter

conventional chemotherapy and radiation therapy resistance and sensitivity in both normal and cancerous tissues in order to enhance the therapeutic index [11,12].

Rationale for Transcriptional Targeting in Cancer Gene Therapy

Clinical trials have confirmed the safety and feasibility of a variety of the gene therapy approaches in the context of ovarian cancer [3,13-21] . Commonly employed vector systems utilized to deliver therapeutic genes to tumor cells include retroviruses, adenoviruses, adeno-associated viruses, and liposomes [22]. However, correlative laboratory studies have demonstrated the limited ability of current generation vector systems to efficiently transduce ovarian tumor cells [14,17]. In addition, limitations in vector specificity can lead to transduction of normal cells and untoward toxicity, even in the setting of compartmental dosing [23].

The success of cancer gene therapy depends on its ability to achieve a high therapeutic index. This requires transgene expression in the tumor, which we have denoted as the "on" status, and absence of expression in the relevant normal tissues, which we have denoted as the "off" status. This issue will be of increasing importance, as high efficient gene transfer vectors and effector systems are developed.

Two strategies have been pursued to achieve tumor-selective gene expression: Transductional targeting is accomplished by modification of vector tropism [24]. Alternatively, gene therapy targeting may be achieved by regulation of transgene expression, denoted as transcriptional targeting. Transcriptional targeting is of premier

interest in tumor types for which specific cell surface markers are not described or do not exist. In this case, and for concepts like targeting of the hypoxic core of solid tumors (see below), transductional targeting is not possible. Furthermore, a combination of transcriptional and transductional targeting might synergistically increase the therapeutic index of corresponding gene therapeutic regimens.

The Concept of Tumor-Specific Promoters

To date, vectors employed for cancer gene therapy have included constitutively active promoters such as the *cytomegalovirus promoter* (CMVp). CMVp is a strongly positive regulator but lacks expression specificity. An additional drawback of the CMVp derives from the fact that it represents a viral sequence, and thus, is frequently downregulated in vivo [25]. As a result, cellular promoters are being explored for cancer gene therapy aiming at specific and persistent expression of therapeutic genes in tumors [26,27].

Most malignant cells retain the capacity to synthesize proteins that are specifically produced in the nonmalignant cell of origin. Several of these proteins are upregulated in tumors. Other proteins are switched on during tumorigenesis, whereby they are specifically expressed in tumor cells and prevalent among the diverse neoplastic cell populations. Gene regulatory elements which drive transcription of these proteins have the potential capacity to control gene expression in a tumor cell-specific manner. Additionally, promoters specifically active in cells of the tumor stroma, like tumor

endothelial cells, or promoters induced by the unique tumor physiology, like hypoxia, show tumor-specific activity. Transcriptional targeting is based on the use of these tissue or tumor-specific promoters (TSPs) in a heterologous context to direct the expression of therapeutic genes specifically to the tumor. A further strategy is to apply treatment-inducible promoters to express gene products that sensitize tumor cells to the inducing therapeutic regimen resulting in a synergistic anti-tumor effect.

The elucidation of TSPs for cancer gene therapy requires definition of the promoter sequences responsible for specific activity. Therefore, deletion analyses of potential regulatory sequences are performed with reporter genes like GFP and LacZ (determination of the fraction of transduced cells) or luciferase (quantification of reporter activity). For this purpose promoter sequences are cloned from genomic DNA, incorporated into the vector of choice and transduced into target and non-target cells. It should be noted that both activity and specificity of a candidate promoter might vary between different vectors. Promoter specificity is derived from the reporter activity in target versus non-target cells relative to constructs with ubiquitous transcriptional control. Subsequent to reporter analysis, efficacy and toxicity studies are performed in vitro and in vivo with constructs containing the candidate promoter driving a therapeutic gene. These studies result in the determination of the therapeutic index of the targeted construct.

Several promoters have been explored for gene therapy in a variety of cancer cell types. For example, the *alpha-feto protein* (AFP) promoter has been used to drive gene expression in hepatic carcinoma cells [28], the *tyrosinase* promoter in melanoma cells [29], the *prostate specific antigen* (PSA) promoter in prostate cancer cells [30], and the

carcinoembrionic antigen (CEA) promoter in adenocarcinomas [31,32]. The results of these studies have demonstrated the feasibility of using TSPs for targeting of cancer gene therapy in various cancer cell types. Most important, selective transcription and reduced side effects of TSP constructs compared to constructs with constitutive promoters have been shown in animal models [33]. Initial clinical gene therapy studies involving transcriptionally targeted gene expression are on-going [34,35].

Ovarian Cancer Cell Specific Promoters for Gene Therapy

There have been several candidate promoters analyzed in gene therapy studies for specific transcriptional control in ovarian cancer cells. For example, tumor specific activation has been shown after infection with a retrovirus encoding the Diphteria toxin A chain gene under the control of the *human chorionic gonadotropin promoter* (hCGp). No expression of the severely toxic transgene was observed in normal ovarian cells and fibroblasts [36].

The *secretory leukoprotease inhibitor* (SLPI) gene has been shown to be expressed in ovarian carcinoma cells, as well as, lung, breast, oropharyngeal, bladder, endometrial, cervical and colorectal carcinomas. The SLPI promoter has been used in a plasmid construct to direct the expression of HSV-tk in a variety of carcinoma cell lines, including those of ovarian origin, i.e. SKOV3 cells, and has achieved specific cell killing [37,38].

High affinity folate receptors are expressed in normal ovaries, and in the vast majority of ovarian adenocarcinomas. The *human alpha folate receptor* gene (HAFR) contains two tissue-specific promoters, P1 and P4. Cowan et al. constructed a recombinant adenovirus that harbored the P1 promoter driving the luciferase gene. Several ovarian carcinoma cell lines were infected, and correlation was demonstrated between folate receptor levels and reporter gene expression. A new adenovirus is currently under construction with this promoter and the *E.coli* CD suicide gene [39].

The *MUC1/DF3* gene encodes the polymorphic epithelial mucin (PEM), which is expressed in human glandular epithelial. This protein is overexpressed in most carcinomas, mainly due to transcriptional upregulation. The cancer associated mucin, although very similar to its normal counterpart, has a distinct antigenic profile [40]. It has been reported that the epitope is expressed in ovarian adenocarcinomas, including serous, mucinous, endometroid, clear cell, and undifferentiated subtypes [41]. Regulatory regions, with distal enhancer elements, have been identified [42]. Ring et al. generated recombinant retroviruses containing the HSV-tk gene under the control of the MUC1 promoter, and showed increased ganciclovir sensitivity in pancreatic and breast carcinoma cell lines. Adenoviruses encoding the MUC1 promoter have achieved specific tumor gene expression in breast, pancreatic and cholangiocarcinoma cells [43]. Lastly, an adenovirus with MUC1 promoter driving the proapoptotic bax gene has shown specific cell killing in ovarian cancer cell lines and in a murine model of ovarian cancer [44].

L-plastin is a member of the actin-binding proteins and is highly expressed in most human epithelial cancer cells [45]. The L-plastin promoter has been incorporated in

a replication deficient adenovirus, driving the *E.coli* LacZ gene. This construct has been tested in a variety of cell lines, including ovarian cancer cells and, mesothelial cells. The L-plastin driven transgene expression appeared to be restricted to the ovarian carcinoma cells, while sparing the mesothelium [46]. Cytotoxicity in ovarian cancer cell lines was achieved with the *E.coli* CD gene replacing the LacZ reporter gene and 5-FC [47].

Thus, there appear to be several candidate promoters poised to be incorporated into clinical trials investigating novel gene therapy approaches in patients with ovarian cancer. The challenge for these trials will be in determining whether controlling transcription via TSPs will enhance the therapeutic index.

Table 1: TSPs that have been employed in OVCA

Promoter	**Tissue specificity**	**Vector**	**Transgene**	**Tested Substrate**
hGC	Ovary	Retrovirus [36]	Dipht. Toxin A	Ovarian cancer cell lines
SLPI	Various adenocarcinomas	Plasmid [37,38]	HSV-tk	Ovarian cancer cell lines
HAFR P1	Ovary	Adenovirus [39]	Luc	Ovarian cancer cell lines
DF3/MUC1	Various adenocarcinomas	Adenovirus [44] Adenovirus [44]	LacZ Bax	Ovarian cancer cell lines and mouse i.p model
L-plastin	Most human carcinomas	Adenovirus [46]	LacZ	Ovarian cancer cell lines and primaries,

		Adenovirus [47] Adenovirus [48]	CD E1A	mouse i.p model Ovarian cancer cell lines Ovarian cancer cell lines
Metallo-thionein	Cisplatin-resistant cells	Liposomes [49]	HSV-tk	Ovarian cancer cell lines

Potential Candidate Targets for Transcriptional Targeting in Ovarian Cancer Gene Therapy

Opportunities for novel transcriptional targeting approaches may result from our increasing knowledge of the molecular biology of ovarian cancer, particularly in regard to overexpressed oncogenes and growth factors that accompany malignant transformation. The most well described oncogenes in ovarian cancer have been growth factor receptors of the *erbB* family and the cell signaling related *ras* proteins [50,51]. Additionally, alterations in expression of the epidermal growth factor receptor, the fibroblast growth factor receptor, PIK3, akt2, fms have been identified in ovarian carcinoma cells [52,53]. Finally, telomerase, the polymerase that catalyzes the expansion of telomeres, has been detected in more than 90 % of ovarian carcinomas [51,54,55]. Each of these overexpressed genes allow opportunities to target related promoters that would control transgene expression in normal and malignant cells.

Midkine is a heparin-binding, growth and differentiation factor, highly expressed in many malignant tumors. Specifically, the midkine gene has been shown to be overexpressed in most ovarian tumors [56]. Velculescu et al. performed serial analysis of gene expression (SAGE) of a variety of tumors versus and their normal counterpart tissues. Although not evaluated in ovarian cancer, midkine gene showed the second highest ratio of tumor/normal tissue expression, [57]. An adenoviral vector with the MK promoter, upstream from the HSV-tk gene, has been tested systemically in a mouse model, together with GCV treatment. It was shown to exhibit a very low profile of liver activity, and a higher therapeutic index than a similar adenovirus encoding the CMV promoter [33].

The *CA 125* antigen is elevated in 80 to 96% of the epithelial tumors of the ovary [58]. Secretion of CA125 appears to be directly linked to the epithelial growth factor receptor signal transduction pathway [59]. However, its potential benefit for transcriptional targeting has not been investigated, as the gene has not yet been characterized [60]. Additionally, normal mesothelial cells have the capacity to secrete CA 125 and may limit specificity of targeting [61,62].

Other potential transcriptional targets include the *hyaluronan receptor* which has been reported to be expressed in 40 % of epithelial ovarian cancers [63]. Expression of *endothelin 1* (ET-1) and *endothelin A receptor* (ET-AR) have been reported in over 90 % and 84% of ovarian cancer patients, respectively [64], and a plasmid encoding the ET-AR promoter and the luciferase reporter gene has been tested in Chinese hamster ovary cells [65]. *Mesothelin* is a surface glycoprotein that is expressed in ovarian cancers. However,

it is constitutively expressed as well in mesothelial cells, hindering its utility for ovarian transcriptional targeting [66-68].

The tumor-associated trypsin inhibitor, a potential marker for ovarian cancer, is mainly expressed in mucinous subtypes [69]. The matrix metalloprotease pump-1 (MMP-7) is also frequently overexpressed in ovarian tumors [70]. Other genes, involved in tumor invasion and angiogenesis, are being increasingly elucidated in the context of ovarian cancer. For example, the urokinase-type plasminogen activator (uPA) and its receptor (uPAR), implicated in the cleavage of extracellular matrix proteins, are elevated in ovarian carcinoma cells [71].

The enzyme *cyclooxygenase* (Cox), that synthesizes prostaglandins from arachidonic acid, has two isoforms, Cox-1 a constitutive form-, and Cox-2, an inducible form associated with cellular growth and inflammatory processes. Cox-2 protein has shown to be highly expressed in a number of epithelial tumors [72,73]. Cox-1 has been proposed as a new ovarian cancer marker [74].

Transcriptional Targeting of Tumor Stroma and Tumor Physiology; Therapy-induced Transcription

Gene expression in ovarian cancer can be transcriptionally targeted at several levels. First, therapeutic interventions can be guided through the recognition of molecular changes that are specific to the tumor cell. Second, the tumor vasculature can be abrogated. This is an attractive substrate, as the endothelium is not prone to mutation-

related resistance. In addition, neovascularization is a common requirement for tumor growth, invasion and metastasis. Third, promoters induced by physiological conditions unique to the tumor might be applied. Lastly, treatment-responsive promoters can be constructed that demonstrate enhanced activity when exposed to conventional therapy.

The vascular endothelial growth factor (VEGF) and its receptors flt-1 and KDR are strongly expressed in most ovarian cancer tumors [75]. Thus, opportunities may exist in designing TSPs that are directed at genes controlling tumor angiogenesis in ovarian cancer. For instance, endothelial cell-specific expression of tumor necrosis factor-alpha has been achieved after infection with retroviral constructs encoding the KDR or E-selectin promoters [76].

An alternative transcriptional strategy to target refractory tumor cells capitalizes on the use of promoters that are induced under certain conditions present in the tumor environment, (such as ischemia) that hinder the efficacy of chemotherapy and radiotherapy. One such TSP is the promoter of the lactate dehydrogenase gene which is efficiently induced by the neighboring hypoxia-response enhancer (HRE) in ovarian cancer cell lines [77].

Several authors have identified specific molecular changes in ovarian cancer (i.e. increased 1q21 and 13q12 copy number) that correlate with resistance to chemotherapy. If these changes are present specifically in the tumor, and absent in the normal tissues, its molecular identification can drive gene therapy co-adjuvant treatments towards refractory cancer cell subpopulations. For example, Vandier et al. have used successfully a plasmid encoding the metallothionein promoter to drive HSV-tk expression to kill ovarian carcinoma cisplatin resistant cells [49]. A retroviral vector, employing this promoter to

express transforming growth factor beta 1 has been used in Chinese ovarian cancer cells [78]. Concomitant therapy may also modulate the activity of target promoters. We have observed, for example (data not published), that certain chemotherapy drugs can induce one promoter and shut off others. In the same way it has been reported that the mdr1 promoter can be induced after chemotherapy [79].

Table 2. Novel transcriptional targets for ovarian cancer

Promoter	**Target**	**Rational**
erbB-2	Many carcinomas	Most frequently expressed growth factor in Ovarian carcinoma (OVCA) [80]. Engineered in retrovirus [40,81] and adenovirus [40,43,82] constructs.
EGFR (erbB1)	Many carcinomas	Expressed in some OVCA[53,83,84]
c-myc	Many tumors	Frequently amplified or overexpressed in OVCA[53,85-87]
Cyclin D1	Many tumors	Overexpressed or amplified in some OVCA [88-92]
hTERT	Carcinomas and germ cell tumors	Telomerase is expressed in most of OVCA [93-97]. A plasmid construct driving caspase 8 gene has shown efficacy for cancer gene therapy [98]
Midkine	Many carcinomas	

ET-1 and *ET_AR*	Endothelium	Both are overexpressed in most OVCA [64]
uPA	Many tumors	UPA and its receptor are frequently elevated in OVCA[71,99]. Also, an X-ray responsive element have been identified in the promoter of the tissue plasminogen activator (TPA) [100]
Inhibin/Activin	Ovary	Elevated expression in ovarian adenocarcinomas[101-105]
Cox1 and *cox2*	Many carcinomas	
E-selectin	Endothelium	Highly expressed in OVCA. It has been used in a retroviral vector driving TNFalpha [76].
KDR/VEGFR	Endothelium	Highly expressed in OVCA. It has been used in a retroviral vector driving TNFalpha [76]
VEGF	Many tumors	VEGF seems to be strongly expressed in most OVCA tumors[106-109]
LDHA-HRE	Ischemia	It has been reported that ischemia induces this promoter in OVCA cell lines [77]

Transcriptional Targeting of Viral Replication

In addition to regulating transgene expression, controlling replication of lytic viruses, including adenovirus and HSV, may also achieve the goals of tumor targeting in ovarian cancer gene therapy. In this new concept the transcriptionally targeted expression of essential viral genes results in an oncolytic virus specifically replicating in the tumor [110]. Recently, Zhang et al. have generated a conditionally replicative adenovirus by placing the EIA gene (necessary for viral replication) under the control of the L-plastin promoter, resulting in tumor specific replication competency [111].

Conclusions and Outlook

Gene therapy is in its infancy with respect to its development as a therapeutic paradigm for ovarian cancer. Clinical trials over the past decade have begun to identify both the feasibility and the limitations of this approach. Incorporating the concept of transcriptional targeting will further refine the methodology, enhance the therapeutic index and highlight the applicability of gene therapy approaches to treat ovarian cancer.

New high throughput differential display technologies [66,112] and the recently completed sequencing of the human genome will result in the defining of a plethora of new target molecules for ovarian cancer. Furthermore, several recent studies focus on optimizing promoter elements or developing artificial promoters aiming at increased activity and specificity of transcriptional control for tumor targeting in gene therapy [26]. These new endeavors will result in the development of new promoters with increased fidelity for ovarian cancer gene therapy.

Acknowledgments

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Article Precis

This manuscript provides an overview of transcriptional targeting strategies utilizing tumor-specific promoters (TSPs) to increase therapeutic specificity and efficiency of human ovarian cancer gene therapy.

Midkine and cyclooxygenase-2 promoters are promising for adenoviral vector gene delivery of pancreatic carcinoma

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Abstract

Midkine (MK), a heparin binding growth factor, and cyclooxygenase-2 (COX-2), a key enzyme in the conversion of arachidonic acid to prostaglandin, are both upregulated at the mRNA or protein level in many human malignant tumors. Here we investigated the tumor-specificity of both MK and COX-2 promoters in human pancreatic cancer, with the aim to improve the selectivity of therapeutic gene expression. Therefore, we developed recombinant adenoviral vectors containing either the luciferase (Luc) reporter gene under the control of the COX-2 or MK promoter or the herpes simplex virus thymidine kinase (Tk) gene under the control of the COX-2 promoter and compared the expression with the cytomegalovirus (CMV) promoter. AdMKLuc achieved moderate to relatively high activity upon infection to both primary and established pancreatic carcinoma cells, as compared to AdCMVLuc activity. Of the two COX-2 promoter regions (COX-2M and COX-2L), both revealed a high activity in primary pancreatic carcinoma cells, while in the established pancreatic carcinoma cell lines COX-2L has an approximately equal high activity as compared to CMV. In addition, both AdCOX-2M Tk and AdCOX-2L Tk induced marked cell death in response to ganciclovir (GCV) in three out of four established pancreatic carcinoma cell lines. From these results, and because it has been reported that AdMKTk (Adachi *et al.*, 2000) and AdCOX-2L Tk in combination with GCV did not reveal significant liver toxicity, we conclude that the MK as well as the COX-2 promoters are promising tumor-specific promoters for adenoviral vector based gene therapy of pancreatic cancer.

Introduction

Pancreatic cancer is the fifth leading cause of cancer death in the Western world. Despite improvement in operative mortality rates, little impact has been made on the overall 5-year survival (1). Conventional chemotherapeutic intervention appears to be largely ineffective in the treatment of pancreatic cancer and there is only marginal survival benefit from the latest generation of drugs (1). Therefore, development of new therapeutic modalities such as gene therapy are necessary to improve patient outcome and serve as a more effective treatment for pancreatic cancer.

Adenoviral (Ad) vectors have been used for both *in vitro* and *in vivo* gene delivery of pancreatic cancer (2,3). However, as observed for other tumor tissue types, a major concern associated with using Ad vectors in pancreatic carcinoma cells is the relatively limited transduction efficiencies achieved *in vitro* (2). One of the approaches to overcome the inefficient delivery to pancreatic carcinoma cells is the use of retargeted Ad vectors which bind to alternative cellular receptors and have been endeavored as a means to improve its specificity and efficacy (4, 5 and Wesseling *et al.*, submitted). In this study we wanted to develop another system to express therapeutic genes in pancreatic carcinoma cells using Ad vectors with a high selectivity for cancer gene therapy. To achieve this, we have been seeking promoters with both tumor specificity in pancreatic carcinoma and minimal transcriptional activity in the liver. We believe this is important, since the liver is the predominant site of Ad vector localization after systemic administration (6), and as a consequence is at risk when Ad vectors ectopically expresses therapeutic genes localized to this site. Whereas a number of promoters have been explored in the context of gene therapy for pancreatic cancer, i.e. the carcinoembryonic antigen promoter (7), few exhibit the optimal profile of inductivity and specificity in pancreatic carcinoma.

Recently it became known that two proteins, the heparin-binding, growth/ differentiation factor midkine (MK) and the enzyme cyclooxygenase-2 (COX-2) appeared to be expressed in high levels in several types of human cancer (8, 9). MK, is a product of a retinoic acid-responsive gene and belongs to the new family of heparin-binding growth/differentiation factors that is induced by retionic acid in embryonal carcinoma cells (8). MK is expressed at higher levels in a number of human tumors, such as Wilms' tumours (10), but also in breast (11), hepatocellular, gastric, pancreatic and colon carcinomas (12), as compared to corresponding normal tissues. Of interest, the human MK promoter has been cloned and identified (13) and appeared to be a candidate tumor-specific promoter for Wilms' tumor or neuroblastoma (14), whereas no expression is observed in human liver (10, 15). The other protein of interest, COX-2, is the key enzyme in the conversion of arachidonic acid to prostaglandins and other eicosanoids and its action is inhibited by non-steroidal anti-inflammatory drugs (16). In resting cells, the expression of COX-2 is usually undetectable, but can be rapidly induced by mitogenic stimuli (serum, phorbol esters, growth factors) as well as inflammatory agents (16, 17). Recent studies have revealed that COX-2 expression is upregulated in a variety of human cancers, including colon (9), gastric (18), esophageal (19) and pancreatic cancer (20). The 5' control region of the COX-2 gene has been cloned and identified (21,22) and it will be of interest to investigate its putative role as tumor-specific element.

On this basis we wanted to investigate whether COX-2 as well as MK are potential candidate promoters for specific activity in pancreatic tumors and compare it with the activity of the cytomegalovirus (CMV) promoter. Therefore, we inserted two regions of the COX-2 promoter as well as one region of the MK promoter into the deleted E1-region of the Ad5 vector and used to drive expression of either the luciferase (Luc) reporter or herpes simplex virus thymidine kinase (HSV Tk) genes. In this way we wanted to test the usefulness of these

two regions as candidate tumor-specific promoters for an adenoviral vector-based cancer gene therapy approach in both primary and established pancreatic carcinoma cells.

Material & Methods

Tumor cells

The established human pancreatic carcinoma cell lines (BxPC-3, Capan-1, Hs766-T and MIA PaCa-2; > 20 passages) were purchased from Boehringer Ingelheim, Belgium. The low passage (primary) human pancreatic carcinoma cells (p6.3 and p10.5; < 5 passages) were obtained from Dr. E. Jaffee, Johns Hopkins University School of Medicine, Baltimore. KATO III cells (gastric carcinoma) were purchased from the American Type Culture Collection (Manassas, VA). KATO III cells were cultured with RPMI 1640 (Mediatech, Herndon, VA) with 20% Fetal Bovine Serum (FBS; Summit Biotechnology, Ft. Collins, CO), 1% L-glutamine and 1% penicillin/streptomycin (Life Technologies Inc., Rockville, MD) streptomycin at 37°C in 5% carbon dioxide atmosphere at 95% humidity. The other cells were cultured at the same conditions, but in this case Dulbecco's minimal essential medium (DMEM) (Mediatech, Herndon, VA) with 10% FBS is used.

Adenovirus vectors

The E1$^-$, E3-deleted adenovirus vector expressing the firefly luciferase gene from the cytomegalovirus (CMV) immediate early promoter, AdCMVluc (23) was obtained from Dr. R. Gerard (University of Leuven, Leuven, Belgium). AdCMV-HSV-Tk, expressing the herpes

simplex virus thymidine kinase gene, was constructed as described earlier (24). AdCMV GFP was obtained from Dr. Parameshwar, Gene Therapy Center, University of Alabama, Birmingham. The human MK promoter region containing 27 bp of exon 1 and 2285 bp of the 5' flanking region of the human MK gene was cloned into Ad vectors, as described (14). Two control regions of the COX-2 promoters were cloned into Ad vectors: the cox-2 M region (from –883 bp to +59 bp) and the cox-2 L region (from –1432 bp to +59 bp), as provided by Drs. Inoue and Tanabe at National Cardiovascular Center Research Institute, Japan (21,22). The recombinant Ad vectors AdCOX-2M Luc and AdCOX-2L Luc as well as AdCOX-2M Tk and AdCOX-2L Tk, under the control of human COX-2M and COX-2L promoter regions, respectively, and the recombinant Ad vectors AdMKLuc, under the control of the MK promoter, were constructed using the "AdEasy" method, reported previously (25). Briefly, the COX-2M, COX-2L and MK promoters (14) with the luciferase gene (pGL3 basic vector, Promega) and the COX-2M and COX-2L promoters with the Tk gene (24) were inserted into a multiple cloning site in the pShuttle vector. The resultant plasmid was linearized with PmeI digestion and subsequently cotransfected into E.coli BJ5183 with pAdEasy-1 adenoviral backbone plasmid. After selection of recombinants in these bacteria, the recombinant of interest were grown up and linearized with PacI digestion and transfected into 293 cells to generate AdCOX-2M Luc, AdCOX-2L Luc, AdCOX-2M Tk, AdCOX-2L Tk and AdMKLuc. The recombinant adenoviruses were propagated in 293 cells, purified by double CsCl density centrifugation and virus titers were determined by plaque assay, as described (26).

Adenovirus vector-mediated gene transfer.

To assess adenovirus-infection efficiency $5x10^4$ of each of the tumor cells per well were plated in 24-well plates and allowed to adhere overnight. The next day the cells were infected with AdCOX-2M Luc, AdCOX-2L Luc, AdMKLuc or AdCMVLuc at a multiplicity of

infection (MOI) of 1, 10 and 100 per cell in phosphate buffered saline (PBS) for 1 h at 37°C. Cell were incubated in PBS without virus as a control. The virus was removed and the cells were incubated for 48 hrs in complete media. The infected cells were harvested and treated with 100 µl lysis buffer. A luciferase assay (Luciferase Assay System, Promega Corp., Madison, WI) and a Berthold luminometer (Lumat, Wallc Inc.) were used for the evaluation of luciferase activities of Ad infected cells. Luciferase activities were normalized by the protein concentration in the cell lysate using the BCA Protein Assay procedure (Pierce, San Fransisco, CA), as described by the manufacturer.

Ad-thymidine kinase (Tk) mediated gene transfer

Tumor cells were plated in 96 well plates in triplicate at a density of 3000 cells per well. After overnight culture, cells were infected with AdCOX-2M Tk, AdCOX-2L Tk, AdCMVTk and AdCMVGFP as a control at an MOI of 500 during 5 hrs. The viral infection was followed by medium replacement including various concentrations of ganciclovir (GCV) ranging from 0 to 10000 µM. The number of surviving cells was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega) after 5 days exposure of GCV using an automated E max spectrophotometric plate reader (Molecular Device Corp., Sunnyval, CA). The depicted results are the mean of triplicate assays and the standard deviation was assessed on basis of the three determinations.

Results

The midkine promoter region in adenoviral context has high activity in pancreatic carcinoma

From previous studies it was confirmed that the MK promoter showed relatively high activity in cells from Wilms' tumors (G-401) in a plasmid context (27) and upon infection with recombinant Ad vectors containing the MK promoter (14). Since such a promoter with both tumor specificity in human pancreatic carcinoma and minimal activity in the liver would be ideal for gene therapy of pancreatic cancer, we thus investigated whether the MK promoter in the adenoviral context would manifest high transcriptional efficiency in both primary as well as in established pancreatic carcinoma cells. To this end, in a reporter gene experiment with firefly luciferase, primary and established human pancreatic carcinoma cells were infected with AdMKLuc or AdCMVLuc at MOIs of 1, 10 and 100 (Fig. 1). In both primary pancreatic carcinoma cells (p6.3 and p10.5) luciferase activity induced by AdMKLuc revealed comparatively high activity, approximately 30% to 70% of the activity induced by AdCMVLuc infection at MOIs of 1 and 10 and approximately 10% of the activity induced by AdCMVLuc at an MOI of 100 (Fig. 1A and 1B). In the four established pancreatic carcinoma cell lines the luciferase activity induced by AdMKLuc showed comparatively moderate to high activity, approximately 10% of the activity induced by AdCMVLuc for the Capan-1 cells, approximately 25% of the activity induced by AdCMVLuc for the BxPC-3 and MIA PaCa-2 cells and approximately 85% of the activity induced by AdCMVLuc for the Hs766-T cells (Fig. 1C – 1F). As a negative control the colon cancer cell line LS174T was infected with AdMKLuc showing less than 1% luciferase activity as compared with AdCMVLuc (results not shown). The data depicted in Fig. 1 thus revealed that the MK promoter is an attractive candidate for tumor-specific gene delivery in pancreatic cancer, as has been

reported for pediatric tumors, with the ultimate advantage of strongly reduced activity in the liver (14).

Cyclooxygenase-2 promoter regions in adenoviral context have high activity in pancreatic carcinoma

Since the cyclooxygenase-2 gene expression has been shown to be upregulated in human pancreatic carcinoma cells (20), we sought to investigate whether COX-2 promoter regions in the adenoviral context would manifest high transcriptional efficiency in primary and in established pancreatic carcinoma cells. Although three major control regions of the COX-2 promoter (NF-κB, NFIL-6 and CRE) exist within 300 bps from the transcription initation site (21,22), we decided to use two longer control regions (COX-2M (-883 bp to +59 bp) and COX-2L (-1432 bp to +59 bp)) to obtain more fidelity. In a reporter gene experiment with firefly luciferase, primary and established human pancreatic carcinoma cells were infected with AdCOX-2M Luc, AdCOX-2L Luc or AdCMVLuc at MOIs of 1, 10 and 100 (Fig. 2). In the primary pancreatic carcinoma cells (p6.3 and p10.5) luciferase activity induced by both AdCOX-2M as well as AdCOX-2L revealed high activity, approximately the same activity as induced by the AdCMVLuc infection. Interestingly, virtual no difference of this high luciferase activity between the two COX-2 promoter constructs (COX-2M and COX-2L) is observed (Fig. 2A and 2B). In the four established pancreatic carcinoma cell lines (BxPC-3, Capan-1, Hs 766T and MIA PaCa-2) the luciferase activity induced by AdCOX-2M and AdCOX-2L also showed this high activity, but in this case AdCOX-2L has approximately 50% to 80% of the luciferase activity induced by AdCMVLuc infection, while AdCOX-2M showed 20% to 30% of the activity induced by AdCMVLuc infection (Fig. 2C – 2F). As a negative control gastric cancer cells KATO III were infected with AdCOX-2M and AdCOX-2L revealing less than 1% luciferase activity as compared with AdCMVLuc (results not

shown). In comparison with results of tumor specific promoters published previously (7) the luciferase activity induced by the COX-2 promoter regions in human pancreatic tumors as depicted in Fig. 2 can be regarded as high and, therefore, the use of especially the COX-2L promoter region looks promising for tumor-specific gene therapy approaches in pancreatic carcinoma.

Effect of COX-2 promoter in combination with Tk and GCV in pancreatic carcinoma cells.

To determine whether Ad-mediated infection with the COX-2M Tk and COX-2L Tk gene would render the four pancreatic carcinoma cell lines sensitive to cell killing by ganciclovir (GCV), these cell lines as well as a control tumor cell line (gastric cancer, KATO III) were infected with AdCOX-2M Tk, AdCOX-2L Tk, AdCMVTk or AdCMVGFP at an MOI of 500. The recombinant Ad infection was followed by 5 days of GCV exposure at concentrations ranging from 0 to 10^4 μM after which the number of surviving cells was determined by an MTT assay (Fig. 3). Both AdCOX-2M Tk as well as AdCOX-2L Tk virus vectors successfully induced GCV sensitivity in the BxPC-3 and MIA PaCa-2 pancreatic carcinoma cell lines with IC50 values (the GCV concentration at which 50% cell survival is seen) comparable to those obtained with the AdCMVTk virus (Fig. 3A and 3D). For the pancreatic carcinoma cell line Hs766-T and the gastric cancer cell line KATO III the induced GCV sensitivity by the AdCOX-2M Tk or AdCOX-2L Tk viruses was much less than seen with AdCMV Tk (Fig. 3C and 3E). Although the luciferase study revealed that both the COX-2M and COX-2L promoter activity was comparable with the CMV promoter activity in the Capan-1 cells (Fig. 2D), the GCV sensitivity caused by AdCOX-2M/2L Tk/GCV was very limited, which might be caused by the relative resistance of the Capan-1 cells for GCV (Fig. 3B). The data revealed that AdCOX-2M/2L Tk induced marked cell-death in response to GCV in two out of four pancreatic carcinoma cell lines, suggesting that the COX-2 promoter

in combination with Tk and GCV might be a promising candidate for tumor specific suicide gene therapy of human pancreatic tumors.

Discussion

Human pancreatic carcinoma cells have been shown to be relatively resistant to adenovirus gene transfer (2), but the use of both epidermal growth factor receptor (EGFR) targeted Ad vectors as well as integrin targeted (i.e. RGD (Arg-Gly-Asp)-modified) Ad-vectors have demonstrated enhanced gene transfer to both primary as well as established pancreatic carcinoma cells (4, 5 and Wesseling *et al.*, submitted). To further improve the selectivity of expressing therapeutic genes in pancreatic cancer, in this study we showed for the first time that the MK promoter as well as the two COX-2 promoter regions in the adenoviral backbone revealed tumor specific expression in both primary as well as established human pancreatic carcinoma cells. Furthermore, experiments with the Tk gene driven by the COX-2 promoter regions clearly indicate that this promoter may be an ideal candidate for tumor-specific suicide gene therapy of pancreatic cancer. Also, the low level of expression of the MK promoter in the liver, combined with the lack of liver dysfunction upon AdMKTk/GCV administration, as shown in a previous study by Adachi *et al.* (14), will prevent liver toxicity reported for the AdCMV-Tk/GCV approach (28). Furthermore, in a recent study Yip-Schneider *et al.* revealed that the COX-2 protein expression in primary human pancreatic adenocarcinoma was found to be significantly elevated than in corresponding normal pancreas tissue (29). This suggests that the relatively high levels of expression of COX-2 and MK in pancreatic tumors will have a significantly enhanced therapeutic window compared to the use of viral promoters such as CMV. From the results of our study it appeared that the COX-2L promoter region revealed a more profound tumor-specific activity in pancreatic carcinoma than the COX-2M promoter, which fits nicely with the observed reduced expression of this promoter region in normal human liver (Yamamoto *et al.*, submitted), as has been shown for the MK-promoter in an earlier study (14).

The finding that the COX-2 and MK promoter regions revealed a luciferase activity comparable as induced by the CMV promoter in both primary and in established pancreatic carcinoma cells, is of importance because most tumor-specific promoters that have been proposed for use in gene therapy vectors previously exhibit levels of activity that are much lower than viral promoters (7). This has lead to the development of amplification strategies to enhance the efficiency of these specific but weak promoters (30,31). Furthermore, a number of candidate tumor-specific promoters have lost a significant part of their specificity when cloned in the adenoviral backbone (32), probably due to cis- or trans-acting enhancing elements in the genome of the Ad vector. In this study, however, both MK- and COX-2 promoters retain their activity and specificity in the Ad context.

In summary, this study clearly demonstrates that the COX-2 and MK promoters are two promising candidate tumor-specific promoters, since they reveal low hepatic activity and toxicity, they show high tumor activity in both primary- and established human pancreatic carcinoma cells and they reveal fidelity in the adenoviral backbone. Combined with the targeted Ad vectors as described previously (4, 5), the COX-2 and MK promoters may be ideal candidate tumor-specific promoters for cancer gene therapy of pancreatic tumors in humans.

Acknowledgements

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Figure Legends

Figure 1.

Tumor-specificity of the midkine (MK) promoter in human pancreatic carcinoma cells.

Primary human pancreatic carcinoma cells: p6.3 (A) and p10.5 (B) and established human pancreatic carcinoma cell lines: BxPC-3 (C), Capan-1 (D), Hs 766-T (E) and MIA PaCa-2 (F) were infected with Ad vectors with luciferase gene under control of the human MK promoter containing 27 bp of exon 1 and 2285 bp of the 5'flanking region of the MK gene (AdMKLuc ■) and the CMV promoter (AdCMVLuc □), at an MOI of 1, 10 and 100. After incubation at 37°C for 48 h, the cells were lysed, the protein concentration of the lysates was determined, and the RLU of luciferase/milligram of total cellular protein is shown graphically. The results are the mean of duplicate assays and each point represents the mean and the standard deviation of the two determinations.

Figure 2.

Tumor-specificity of Cox-2 promoter in human pancreatic carcinoma cells.

Primary human pancreatic carcinoma cells: p6.3 (A) and p10.5 (B) and established human pancreatic carcinoma cell lines: BxPC-3 (C), Capan-1 (D), Hs 766-T (E) and MIA PaCa-2 (F) were infected with Ad vectors expressing the luciferase gene under control of two regions of the COX-2 promoter: COX-2M (-883 bp — +59 bp; AdCOX-2M Luc ■) and COX-2L(-1432 bp — +59 bp; AdCOX-2L Luc ▒) and the CMV promoter (AdCMVLuc □), at an MOI of 1, 10 and 100. After incubation at 37°C for 48 h, the cells were lysed, the protein concentration of the lysates was determined, and the RLU of luciferase/milligram of total cellular protein is shown graphically. The results are the mean of duplicate assays. Each point represents the mean and the standard deviation of the two determinations.

Figure 3.

GCV sensitivity of pancreatic carcinoma cell lines transduced with Adcox-2M Tk, AdCOX-2L Tk or AdCMV Tk. Established human pancreatic carcinoma cell lines: BxPC-3 (A), Capan-1 (B), Hs 766-T (C), MIA PaCa-2 (D) and the human gastric cancer cell line KATO III (E) were infected with AdCOX-2M Tk, AdCOX-2L Tk, AdCMV Tk or AdCMVGFP at an MOI of 500 for 5 hours. GCV was applied at the concentration ranging from 0 to 10^4 μM and 5 days later the percentage of surviving cells was calculated from the number of surviving cells without GCV (=100%) and the number of surviving cells at various concentration of GCV. The number of surviving cells was determined by an MTT assay. The results are the mean of triplicate assays.

Figure 1

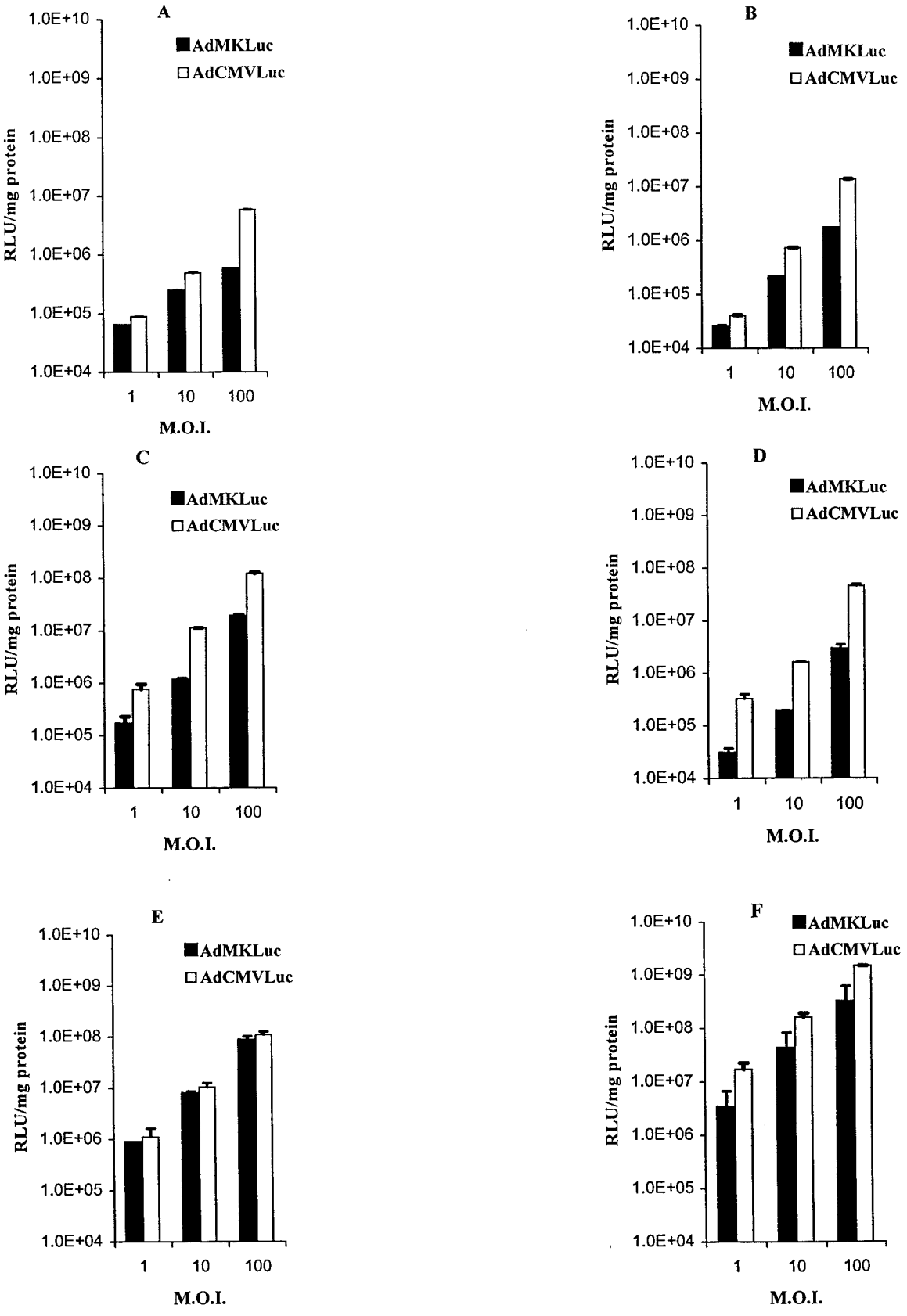
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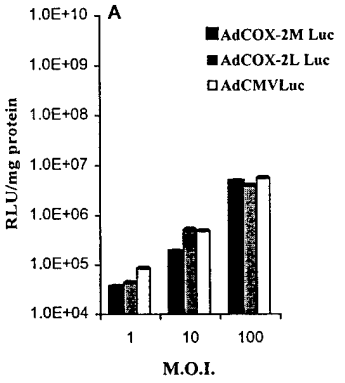
Figure 2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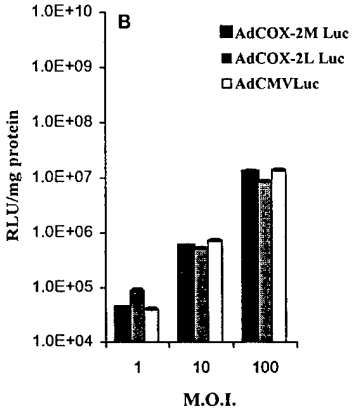
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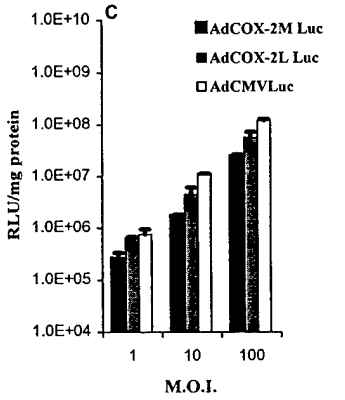
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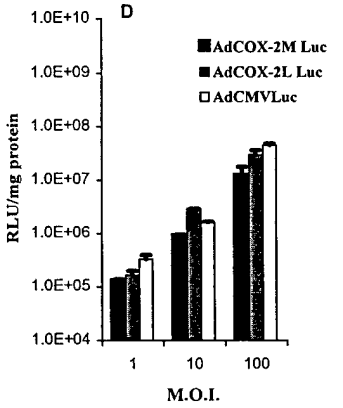
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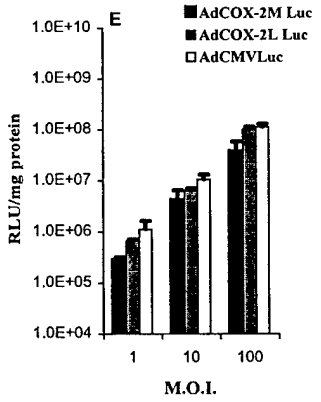
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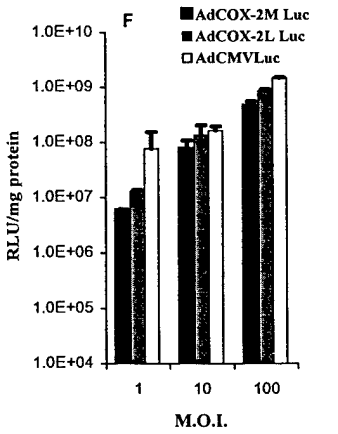
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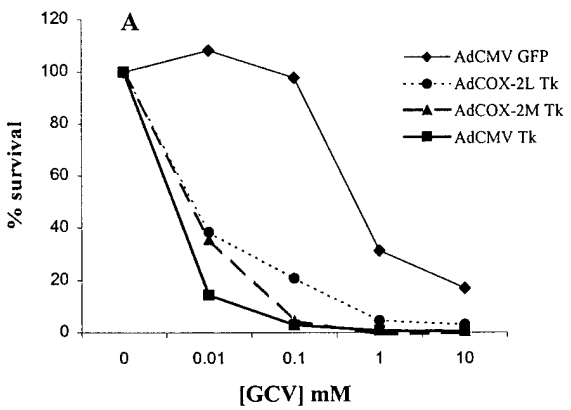
Figure 3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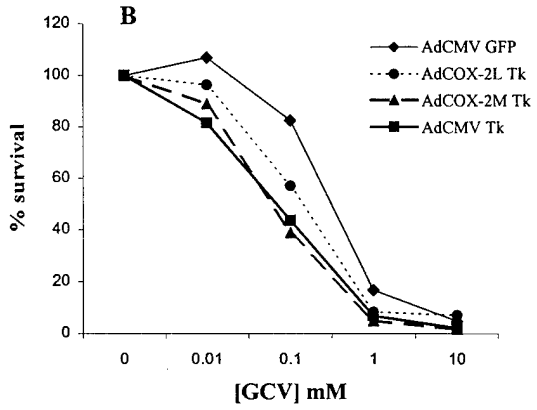
[GCV] mM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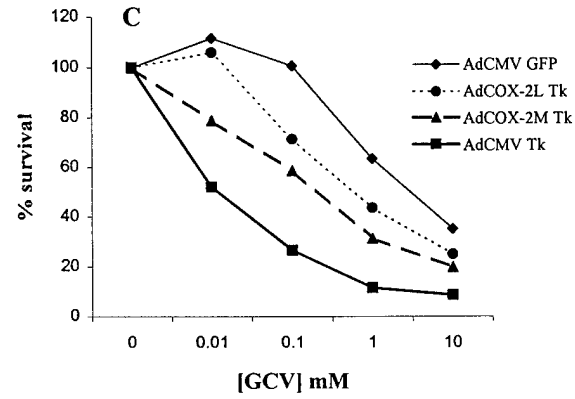
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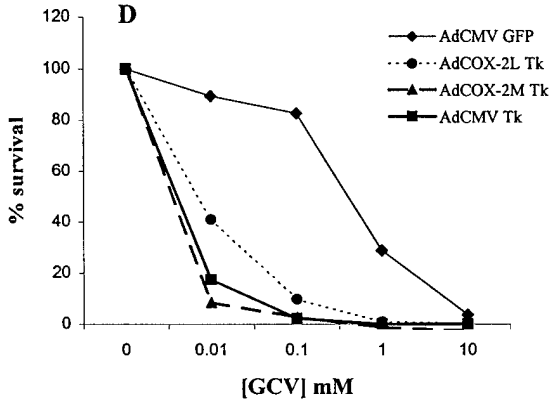
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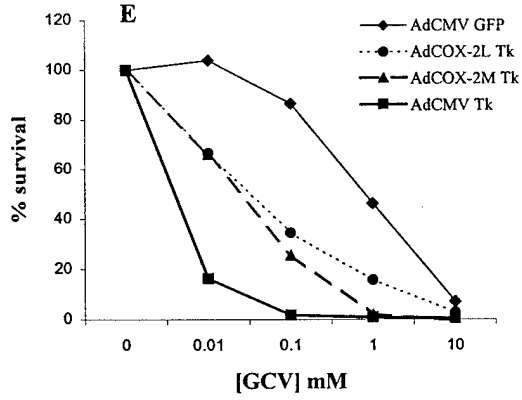
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% survival
AdCMV GFP
AdCOX-2L Tk
AdCOX-2M Tk
AdCMV Tk
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[GCV] mM

Submitted, 2000

Gene Therapy in the Treatment of Human Cancer

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1. Introduction

1.1. Concept

It is well established that most cancers result from a series of accumulated, acquired genetic lesions. To a larger and larger extent, the genetic lesions associated with malignant transformation and progression in a wide variety of human cancers are being identified. In this regard, gene therapy is emerging as a new method of preventive and therapeutic intervention against cancer targeted at the level of cellular gene expression (1). In this approach, altering the complex cancerous pathophysiologic state is achieved by delivering nucleic acids into cells. These nucleic acids may be genes, portions of genes, oligonucleotides, or RNA. In conventional therapeutics, as in pharmacotherapy, altering a cell or tissue phenotype is accomplished by altering cell physiology or metabolism at the level of protein expression. In contrast, in gene therapy this is accomplished by changing the pattern of expression of genes whose products may thus achieve the desired effect on the cellular phenotype.

1.2. Why gene therapy?

In the treatment of human disease, gene therapy strategies may offer the potential to achieve a much higher level of *specificity* of action than conventional drug therapeutics by virtue of the highly specific control and regulatory mechanisms of gene expression that may be targeted. Additionally, interceding at an earlier, upstream step in disease pathogenesis (oncogenes, tumor suppressor genes, and their kin) may offer greater potential to induce *fundamental changes* in phenotypic parameters of disease, with a more favorable clinical outcome. The eventual availability of gene transfer systems, or vectors, for permanent or long-term genetic modification of cells and tissues can allow *definitive* therapeutic or preventive interventions. Furthermore, gene transfer may be accomplished in a limited *loco-regional* context, producing a high concentration of therapeutic molecules in the local area. Thus, undesired systemic effects of those therapeutic molecules are avoided. Lastly, using the body to produce therapeutic proteins, potentially in only certain tissues, has practical advantages of its own (2). Briefly, limitations associated with manufacture, stability, and duration of effect after administration of drugs based on synthetic peptides are completely avoided. From the same

pharmacological point of view, designer drugs based on small molecules, currently under intensive investigation, can hardly be at this point a therapeutic alternative as substitutes for the protein products of tumor suppressor genes.

In the treatment of human malignant tumors, several obstacles explain the limits of currently available treatments for achieving, in most cases of advanced disease, definitive cures (Table 1). It is apparent that new chemotherapy drugs, higher drug doses, cytokines, novel modalities of radiotherapy, and more ambitious and sophisticated surgeries can achieve *incremental* improvements in cancer treatment. But these therapies do not address critical biological obstacles, and thus probably will not bring the much-needed radical advances in the implementation and results of cancer treatment. Gene therapy, in contrast, offers certainly the potential for overcoming some of those fundamental barriers (Table 1).

1.3. Strategies

A number of strategies have been developed to accomplish cancer gene therapy. These approaches include (i) mutation compensation, (ii) molecular chemotherapy, and (iii) genetic immunopotentiation. For mutation compensation, gene therapy techniques are designed to correct the molecular lesions that are etiologic of malignant transformation, or to avoid the contribution by tumor-supporting normal cells. For molecular chemotherapy, methods have been developed to achieve selective delivery or expression of a toxin gene in cancer or tumor stromal cells to induce their eradication. Also, attempts have been made to deliver genetic sequences that protect normal bone marrow cells from the toxic effects of standard chemotherapeutic drugs, thus allowing the administration of higher drug doses without reaching otherwise limiting myelosuppression. Genetic immunopotentiation strategies attempt to achieve active immunization against tumor-associated antigens by gene transfer methodologies. Both tumor cells and cellular components of the immune system have been genetically modified to this end. Importantly, each of these approaches has been rapidly translated into human gene therapy clinical trials (Table 2).

To accomplish any gene therapy approach, certain *basic criteria* must be met to allow an effective genetic intervention. In this regard, gene therapy approaches are based upon the fundamental ability to deliver therapeutic nucleic acids into relevant target cells. Further, the delivered genes must be expressed at an appropriate level and for an adequately prolonged period of time. Finally, the

delivery and expression of the therapeutic genes must not be deleterious to the surrounding normal tissue, nor to the individual as a whole (3). In practice, two general approaches have been employed to met these gene vector criteria: an *ex vivo* approach and an *in vivo* approach. In the former method, target cells are removed from the body and transduced with the genetic vector extracorporally, followed by reimplantation. This approach has allowed reasonably efficacious transduction of target cells and has also allowed for a safety characterization of modified cells, prior to delivery into the patient. Unfortunately, the number and types of parenchymal cells that can be modified in this manner are quite limited.

An alternate approach to achieve therapeutic gene delivery has been the *in vivo* administration of vectors into target parenchymal cells directly in their natural body location. In this regard, both *viral and nonviral vectors* of diverse types have been employed to achieve *in situ* transduction of relevant target parenchymal cells (Table 3). In general, a fundamental recognition in many of these studies, including many clinical trials, has been the disparity noted between the *in vitro* and *in vivo* gene transfer efficiencies of these vector systems, and the suboptimal tumor transduction that presently available vector systems can achieve. In addition, the promiscuous tropism of current vectors may potentially allow for genetic modification of a number of normal tissues besides target cells. Furthermore, this non selective gene transfer impedes the administration of vectors to tumor cells by the systemic route. Thus, important limitations of current approaches used for implementation of gene therapy for cancer have been noted. Although many potentially effective strategies exist to effect the molecular treatment of cancer, *gene delivery issues currently limit the definitive evaluation of these methods.*

In this regard, we examine below the lessons learned from the results of the first attempts to apply gene therapy in human cancer. This will show both the rationale of gene therapy and the problems encountered in its development. We emphasize in our discussion the general biological concepts of each therapeutic strategy, and suggest comprehensive reviews for readers interested in detailed discussions. Finally, we illustrate prospects for overcoming the obstacles to gene therapy by novel methods that are currently being refined.

2. Mutation Compensation

Gene therapy techniques based on mutation compensation are designed to rectify either the molecular lesions in the cancer cell etiologic of malignant transformation, or associated changes in stromal cells that support cancer progression. Many chromosomal changes and mutated genes associated with cancer have been identified, although the exact order of their activation and their precise roles in the progression of cancer have been defined only in a minority of tumor types. However, the elucidation of the molecular basis of carcinogenesis progresses steadily and supports the consideration of genetic approaches for cancer therapy. The genetic lesions involved in the pathogenesis of the malignant transformation may be thought of as a critical compilation of two general types: aberrant expression of 'dominant' oncogenes or loss of expression of 'tumor suppressor' genes. In addition to changes in the individual cancer cells, the contribution of both its local microenvironment and the host are critical during cancer progression. Angiogenesis, cellular mobility, invasion, and metastasis are examples of processes controlled by multiple genes that are involved in carcinogenesis. Gene therapy approaches have been proposed to achieve correction of each of these lesions and processes (Table 4).

2.1. Therapeutic modalities

The knowledge on the major role that growth factors, signaling molecules, cell cycle regulators, and determinant factors of angiogenesis, invasiveness, and metastasis play in neoplastic progression has positive implications for gene therapy. That is, it is possible to abrogate the malignant phenotype by correcting the underexpression of tumor suppressor genes or overexpression of oncogenes. The inactivation of tumor suppressor genes contributes to the neoplastic phenotype by abrogating critical cell cycle checkpoints and DNA repair mechanisms. To approach this loss of function, the logical intervention is replacement of the deficient function with its wild-type counterpart gene. For dominant oncogenes, it is the aberrant expression of the corresponding gene product that elicits the associated neoplastic transformation. In this context, the molecular therapeutic intervention is designed to ablate expression of the dominant oncogene. Inhibition of oncogenic function can be attempted at three levels. First, transcription of the oncogene can be inhibited. This approach uses triplex-forming, antisense oligonucleotides or other sequences that bind transcriptional start sites in the genomic DNA. Second, translation of the oncogene messenger RNA can be blocked, also using antisense sequences, which function by promoting RNAse degradation of the message. Third,

mobilization of the nascent oncoprotein can be blocked or its function can be inhibited when in its final cell location. These strategies involve the use of 'intracellular antibodies' that intercept and interfere with the processing of the oncoprotein, or the heterologous expression of mutant proteins that can inhibit the function of the native oncoprotein, respectively.

2.1.1. Replacement of tumor suppressor genes

Mutations of more than two dozens tumor suppressor genes have been described in numerous cancers. Their functions are diverse and include structure and signaling of intercellular junctions and receptors (*APC*, *DCC*, *DPC4*, *NF1*), components and regulation of the transcription apparatus (*VHL*, *RB1*, *WT1*, *p53*), and DNA mismatch (*hMLH1*, *hMHS2*, *hPSM1*, *hPMS2*) or excision (*XPA*, *XPB*, *XPC*, *XPD*, *XPG*) repair. Of these, *p53*, *RB1*, and *BRCA1* are currently being administered in clinical trials as replacements for the mutated counterparts (Table 4).

The most common genetic alteration found in human cancer involves the *p53* tumor suppressor gene, affecting approximately 50 per cent of all cancers. Besides its high frequency, correction of mutations of *p53* may be particularly relevant due to the central role of *p53* as guardian of the genome and regulator of apoptosis (4). In this regard, *p53*-dependent apoptosis modulates the cytotoxic effects of common antitumor agents such as ionizing radiation and chemotherapy (5). An additional mechanism of action of *p53* could be inhibition of angiogenesis (6; 7). The logical intervention for approaching loss of function of this tumor suppressor gene has been replacement of the deficient function with its wild-type counterpart (8). This general strategy allows phenotypic correction (usually with subsequent apoptosis) both *in vitro* and *in vivo* in a variety of tumors. In particular, several authors have shown, in murine models employing human cancer xenografts, that intratumoral delivery of the wild-type *p53* gene via recombinant viruses can prolong survival by inducing apoptosis in tumor cells (9). Importantly, nontransformed cells can tolerate exogenous administration of *p53*, thereby providing an optimal therapeutic index for this intervention. It must be emphasized that restoration of wild-type *p53* expression in cells with a mutant or deleted gene has been shown to be sufficient to cause apoptosis or growth arrest, despite the presence of multiple additional genetic abnormalities in the tumor cell. This fact has established the rationale for human clinical gene therapy trials designed to achieve mutation compensation through restoration of *p53* in several cancers. In a pioneering study, Roth et al. administered intratumoral injections of a *p53*-encoding retrovirus to patients with nonsmall-cell lung cancer (10). Nine patients with refractory,

mutant *p53*-containing tumors entered the study. Vector sequences were evident in eight of the treated tumors. In addition, apoptosis was observed in six out of seven evaluated tumors. Three patients experienced tumoral regression, and in the other three there was stabilization; none presented toxicity related to the treatment. A second trial is evaluating adenovirus-mediated delivery of *p53* with or without the chemotherapeutic drug cisplatin. Thus, replacement of *p53* is a clinically feasible genetic intervention that can induce tumor regressions *in vivo*.

Replacement of *RB1* and *BRCA1* has shown similar preclinical experimental results, and is also undergoing clinical testing. Attempts to restore wild-type *RB* have been described for prostate, retinoblastoma, osteosarcoma, breast, bladder, and non-small cell lung cancers (11). Of note, and perhaps not surprisingly, some *RB*-deficient tumors have shown persistent tumorigenicity and proliferation after successful restoration and expression of wild-type *RB*, a phenomenon referred to as 'tumor suppressor resistance' (12). Thus, restoration of the *RB* tumor suppressor gene in certain tumors may not effect complete reversion of the malignant phenotype. *BRCA1* is rarely affected in spontaneous tumors, and its function has not been completely characterized. Its clinical use has therefore been somewhat controversial (13).

2.1.2. Inhibition of gene transcription

In addition to mutations that cause the loss of normal tumor suppressor functions, most tumors exhibit dysregulated oncogenes. In particular, dominant oncogenes implicated in cancer and used as targets in clinical trials include genes encoding: (i) growth factors, such as insulin-like growth factor 1 (IGF-1), and transforming growth factor β1 (TGF-β); (ii) growth factor receptors, such as erbB-2; (iii) proteins involved in cell signaling, such as K-ras; (iv) transcription factors, such as c-myc. Modulation of genes encoding cell cycle regulatory proteins, although being tested *in vitro*, has not been employed in human clinical trials yet. In addition, genes involved in a variety of phenotypic characteristics dependent on multiple genes have been described. Examples of these processes are angiogenesis, the development of metastasis, and resistance to chemotherapy.

One possible way of ablating a dominant oncogene is by inhibiting its promoter regulatory DNA sequence. It has been shown, for instance, that the K1 mutant of the viral SV40 large T antigen inhibits the human c-erbB-2 promoter in human ovarian cancer cells. Moreover, liposome-mediated K1 gene transfer decreases the p185 erbB-2 protein level by K1 expression in these cancer cells, and

significantly prolongs survival in an *in vivo* orthotopic animal model (14). Although not a human gene, the E1A gene of adenovirus serotype 5 exhibits tumor suppressor functions in cancer cells overexpressing the oncogene c-erbB-2. Apparently, E1A inhibits transcription of the human c-erbB-2 promoter and accordingly suppresses the tumorigenicity and metastatic potential induced by the oncogene. Studies have shown that both cationic liposomes and an adenoviral vector can efficiently deliver E1A into ovarian tumor cells in mice, resulting in suppression of tumor growth and significantly longer survival of treated animals (15). This findings are the basis for two human gene therapy clinical trials, currently ongoing, that study the intraperitoneal and intratumoral administration, respectively, of a cationic lipid complex containing the E1A gene in patients with breast or ovarian cancer, and other solid tumors overexpressing c-erbB-2.

2.1.3. Antisense

The most universally employed methodology to achieve oncogene ablation, however, is the utilization of 'antisense' molecules, i.e. DNA or RNA oligonucleotides with sequence complementary to that of a nucleic acid target. These molecules are designed to specifically target 'sense' sequences to achieve blockade of the encoded genetic informational flow. Intervention approaches used along this pathway have included the use of 'triplex' DNA to achieve functional ablation of transcriptional activation through blockade of binding sites of transcription factors. This approach has been developed in *in vitro* model systems for targeting the c-*myc*, c-*ras*, and c-*erbB-2* oncogenes. Targeting is also achieved at levels of gene expression distal to transcription. Specific antisense binding to transcribed RNA sequences may interrupt the flow of genetic information through several mechanisms including RNase degradation, and less probably impaired transport, and translational arrest. These interventions may be accomplished by simple antisense oligonucleotides as well as by antisense molecules that possess catalytic activity to accomplish cleavage of target sense sequences, so called 'ribozymes' (16; 17).

A variety of experimental models, both *in vitro* and *in vivo*, have demonstrated the potential utility of the antisense approach as an anti-cancer therapeutic (18-21). Evidence for a specific effect of antisense molecules has been particularly compelling in selected cases, and these molecules are currently undergoing clinical tests. These include antisense sequences against insulin-like growth factor 1 in glioma (22), K-*ras* in lung cancer (23-27), c-*myc* in breast (28) and in prostate cancer (29), and TGFβ in glioma (30-32) (Table 4). The specificity of antisense molecules has been convincingly

shown in the case of K-*ras*. Antisense oligonucleoside methylphosphonates directed at either normal human Ha-ras p21 or ras p21 mutated at a single base in codon 61 have been examined for their efficacy and specificity as inhibitors of p21 expression. Mixed cultures of cells expressing both forms of p21 were treated with the antisense oligomer complementary to the normal p21 or with the antisense oligomer complementary to the point-mutated p21. Each of the antisense oligomers specifically inhibited expression of only the form of ras p21 to which it was completely complementary and left the other form of p21 unaffected. Thus, in general the antisense approach offers the potential to achieve targeted disruption of specific genes in human cancer.

Despite the potentially novel therapeutic strategies offered by the antisense approach, this methodology in practice is associated with *severe limitations* (19; 33). These practical constraints have limited wide employment of this technology in protocols of human anticancer gene therapy. Most disturbingly, there are no universal rules dictating the efficacy of a given antisense oligonucleotide for achieving specific gene inhibition. An array with 1,938 oligonucleotides ranging in length from monomers to 17-mers has been built to measure the potential of the oligonucleotides for heteroduplex formation with rabbit β-globin mRNA (34). The oligonucleotides were complementary to the first 122 bases of the mRNA. Surprisingly, very few oligonucleotides gave significant heteroduplex yield and no obvious features in the mRNA sequence or the predicted secondary structure could explain this variation. In fact, despite the utility of antisense inhibition in selected contexts, attempts to achieve antisense blockage of great many cancer-related genes have failed. In addition, delivery of the antisense molecules has been highly problematic. The tumor environment is deleterious to these unstable molecules, and it is often difficult to maintain effective intracellular levels. To circumvent this problem, a number of design modifications of the antisense molecules have been developed to enhance their *in vivo* stability. In addition, a number of vector approaches have been explored for effective cellular delivery. Despite these various maneuvers, the overriding limitations to the employment of this still promising therapeutic modality remain the idiosyncratic efficacy of specific antisense for a given target gene and the suboptimal delivery of antisense molecules.

2.1.4. Single-chain antibodies

Dominant oncogenes have also been targeted at the level of the protein level. Techniques have been developed to allow the derivation of recombinant molecules that possess antigen-binding

specificities expropriated from immunoglobins (35). In this regard, single-chain immunoglobin (scFv) molecules retain the antigen-binding specificity of the immunoglobin from which they were derived, but lack other functional domains characterizing the parent molecule. The encoded scFv may be expressed in the target cell and localized to specific, targeted subcellular compartments by incorporating appropriate signal molecules. Based on this, a novel approach to oncogene suppression has been developed in our laboratory. It was hypothesized that if an anti-erbB-2 antibody could be localized to the endoplasmic reticulum (ER) of cancer cells, the nascent, newly synthesized erbB-2 protein would be entrapped within the ER and therefore be unable to achieve its normal cell surface localization. It was further hypothesized that this intracellular entrapment would prevent the erbB-2 product, a transmembrane receptor, from interacting with its ligand, thus abrogating the autocrine growth factor loop driving malignant transformation in erbB-2 overexpressing cell lines. We constructed a gene encoding an anti-erbB-2 scFv with a signal peptide sequence that dictates its localization to the ER. The construct was cloned into an eukaryotic expression vector and transfected into the erbB-2 overexpressing ovarian carcinoma cell line SKOV3. We showed that intracellular expression of the anti-erbB-2 results in the following cellular effects: (i) down-regulation of cell surface erbB-2 expression; (ii) marked inhibition of cellular proliferation; (iii) marked reduction in survival of neoplastic cell clones; and (iv) selective cytotoxicity in tumor cells expressing the oncogene target (36). In addition, scFv-mediated erbB-2 ablation induced additional phenotypic alterations in tumor cells, including chemosensitization and radiosensitization (37). In addition, the ability to accomplish selective abrogation of erbB-2 expression was shown in animal treatment models, as well as to transfect, and eradicate primary human ovarian cancer cells (38). This suggests the feasibility of a novel therapeutic approach for tumor cell eradication by utilizing intracellular immunoglobulins to achieve targeted disruption of dominant oncogenes, thereby accomplishing reversion of the malignant phenotype, chemosensitization, radiosensitization, or initiation of cell death. Furthermore, this novel method of oncogene 'knockout' may offer significant practical advantages compared to antisense methodologies, such as the use of monoclonal antibodies already developed against cancer as parental antibodies, and the use of DNA based methods for delivery implicit in the scFv strategy. To this end, we have translated the strategy into an approved human clinical gene therapy protocol for ovarian carcinoma (39). The feasibility of this strategy against the oncogenes *ras* and c-*myc* has also been shown in animal models (40).

2.1.5. Transdominant molecules

Alternatively, oncogenes can be inactivated at the protein level by the heterologous expression of mutant proteins that inhibit the function of the native version of oncoproteins, the so called dominant-negative mutation strategy. For instance, transdominant mutants of H-*ras* have been shown *in vitro* to possess potent suppressive effect on pancreatic cell lines with *ras* mutations. The feasibility of efficient delivery and production of adequate levels of mutant protein *in vivo* remains to be determined.

2.2. Tumor phenomena dependent on multiple genes

2.2.1. Angiogenesis

The development of new blood vessels is a critical factor in the growth, progression, and metastatic spread of both solid and hematopoietic tumors. Despite heterogeneity in many other respects, all tumors share a *universal* feature, i.e. they depend absolutely on the vasculature to maintain their viability and to sustain their growth and dissemination. Extensive experimental data supports this contention (41-44). Furthermore, numerous clinical studies have shown the correlation between the development of intratumoral microvessels and the prognosis of individual cases in a variety of cancers (45-47). Vessel targeting, therefore, should be useful for the treatment of most kinds of cancer (48-51). Importantly in this regard, the genetic stability of endothelial cells should essentially eliminate the appearance of resistance to molecular therapeutic interventions targeted to the endothelium (52). This hypothesis has indeed recently been confirmed in a cancer animal model of treatment with the natural inhibitor of angiogenesis endostatin (53). An additional advantage of targeted killing of endothelial cells is the highly amplified killing effect over large numbers of tumor cells when deprived from its vascularization.

In the last decade, antiangiogenic drugs targeted to the proliferating endothelium of tumors and other diseases have been applied in the clinical setting and have entered clinical trials. In addition, the association of chemo- or radiotherapy with antiangiogenic agents has been shown to produce an enhanced antitumor effect in preclinical models. Notably, combined treatments can achieve cures that are not observed with either treatment alone (54). Thus, molecular therapeutic interventions against the tumor and its vasculature are not only strongly appealing in theoretical grounds for their use in a variety of clinical contexts, but their utility is also rapidly being tested clinically (55). Based

on this, genetic modification of the endothelium of tumor vasculature has been proposed as an alternative therapeutic modality (56). With this genetic strategy, the problems of previously explored approaches can be potentially overcome. For instance, local production of high levels of therapeutic proteins can be induced, thus obviating or diminishing the difficulties associated with systemic toxicity, and also pharmacological issues, such as large-scale manufacture, bioavailability, and cost of ordinary drugs. In addition, the ability to continuously release the gene-encoded product may be relevant in certain cases, such as for the appropriate antiangiogenic effect of interferon γ.

Both suppression of angiogenic cellular signals and augmentation of natural inhibitors of angiogenesis have proved to be feasible strategies in *in vivo* tumor models. Examples of effective genetic interventions for the suppression of angiogenesis factors are the downregulation of vascular endothelial growth factor (VEGF) by antisense molecules, as shown in models of glioma (57; 58), and the blockade of VEGF receptor function by delivery of mutant versions of one of its cognate membrane receptors, Flk-1 (59-61), or of a secreted soluble version of its other receptor, Flt-1 (62 and our own work, Goldman CK et al, *in press*). Conversely, the replacement or supplementation of inhibitors of angiogenesis has been tried by using viral vectors that encode soluble platelet factor 4 (63) and angiostatin. However, no one of these strategies has been clinically tested, and major issues remain still to be solved. Most obvious is the probable need to assure long-term expression of the therapeutic antiangiogenic genes to keep the tumor deprived of its growth-enabling vascularization. In addition, the current lack of targetable, injectable vectors impedes the application of anti-angiogenesis gene-based strategies to multiple foci of tumor that characterize disseminated cancer. Lastly, different combinations of endothelial growth factors and its receptors are altered in different tumors, and may even change in single tumors during different stages of progression. Thus, despite its powerful rationale, the successful clinical implementation of antiangiogenesis gene therapy will require major developments.

2.2.2. Invasion and Metastasis

Increasingly, genes and proteins involved in phenotypic aspects of tumors, other than disordered proliferation, are being described and identified as potentially useful therapeutic targets. In this regard, besides angiogenesis, one fundamental component of the metastatic cascade is the local invasion of the extracellular matrix by tumor cells. Studies in animal models have begun to show

that modulation by gene transfer of molecules involved in degradation of extracellular matrix (ECM), cellular motility, and cellular adhesion has the potential for inhibiting tumor cell spread (64).

Urokinase-type plasminogen activator (uPA) is a protease involved in the processes of tissue remodeling, tumor invasion and cell migration *in vitro*. Plasminogen activators are thought to degrade ECM proteins and cellular basement membranes and to allow local tumor invasion and access to the vascular system for metastasis (65). The inhibitors of plasminogen activation, PA-1 and PA-2, have also been described in association with different types of cancer. The levels of both uPA and its receptor uPAR are elevated in ovarian, prostate, glioma, and other tumor cells and correlate with the clinical stage of disease (66). Furthermore, it has been shown that inhibition of uPA receptor expression by antisense oligonucleotides can abrogate human glioblastoma spread in an *in vitro* model (67). In addition, in an *in vivo* model of uveal melanoma, an adenoviral vector was used for the transfer of plasminogen activator inhibitor type 1 (PAI-1) cDNA (68). Intraocular injection of the vector resulted in a 50% reduction in the number of animals developing liver metastases and a reduction in the metastatic tumor burden in animals that eventually developed metastases. These results support disruption of uPA function through gene transfer as an experimental strategy for preventing metastases and prolonging host survival.

Glioblastomas are known to express the CD44 cell adhesion molecule. Human glioma cell adhesion and invasion *in vitro* may in part be mediated by the interaction of CD44 with extracellular matrix proteins. To suppress the growth and invasive effects of CD44 expression on primary brain tumors, a hammerhead ribozyme against CD44 was designed, and showed significant *in vivo* cleavage activity against cellular CD44 transcripts following transient transfection into a glioma cell line. These results suggest that CD44-directed downregulation may be a useful gene therapeutic maneuver. Therefore, abrogation of molecules involved in tumor cell adhesion may inhibit invasion and represent a novel approach to limiting the spread of locally aggressive tumors.

2.3. Obstacles to mutation compensation

Although the strategies currently used for restoration and ablation of mutant genes have offered in-depth insights into the molecular biology involved in carcinogenesis and tumor progression of cancer, they face critical problems for allowing their clinical application. Human tumors are remarkably heterogeneous in the patterns of expression of relevant oncogenes. Thus, therapeutic

targeting of a single molecular abnormality may have only an inconsequential impact on the clinical management of the disease, both for the population and for individual patients. In addition, several mutated genes produce molecules with transdominant effects, thus requiring the blocking of their effects and not only the mere supplementation with a wild-type version of the gene. Furthermore, because these strategies modulate intracellular responses, nearly every tumor cell must be targeted for these approaches to be clinically effective. The current state of development of gene therapy vectors, both viral and non-viral, makes this feat unachievable within non-toxic margins of vector dose. Clearly, breakthrough developments in vector technology are needed for these obstacles to be overcome. Also, approaches such as molecular chemotherapy or immune system augmentation that exhibit an amplified regional or systemic effect hold the promise of tackling, by their own design, some of the aforementioned limitations.

3. Molecular Chemotherapy

A number of distinct approaches to accomplish molecular chemotherapy for cancer have been developed. These include the administration of (i) toxin genes to eliminate tumor cells and the stromal cells that support them, (ii) of drug resistance genes to protect the bone marrow from myelosuppression induced by chemotherapy, and (iii) of genes that enhance the effect of conventional anticancer treatments. Initially, the approach of molecular chemotherapy was designed to achieve selective eradication of carcinoma cells via expression of a toxin gene. This is similar to conventional chemotherapy, where pharmacological agents are employed. However, in the latter approach, the drug's toxicity is often expressed both in malignant and nonmalignant cells. Therefore, in order to effect a reduction in burden of neoplastic cells, the patient's normal tissues and organs have to be exposed to potentially harmful quantities of the drug. Molecular chemotherapy is designed to circumvent this limitation by selectively targeting toxin delivery or expression into cancer cells on the basis of more specific tissue or transformation-associated markers, and thus reduce the potential for nonspecific toxicity. Commonly, a non-toxic prodrug is administered that requires activation in genetically modified cells to be transformed into a toxic metabolite that ultimately leads to cell death (69-73).

established the necessary biological properties to elicit bystander-mediated cell killing in a murine model of ovarian cancer (81)As a result, a human gene therapy clinical trial has been initiated (82).

In this regard, the basic *biological mechanisms* that underlie the bystander effect have been partially characterized. Both local and distant bystander effects have been described in *in vitro* and *in vivo* models. The local amplification of toxin gene expression includes the transfer of toxic metabolic products of ganciclovir through intercellular gap junctions (83; 84), and, less probably, phagocytosis by live tumor cells of apoptotic vesicles from dead cells (79; 85). Both the local and distant bystander effects, observed *in vivo* in distant foci of untreated tumors, are accompanied by the regional induction of cytokines (86) and an immune cellular response against the tumor (87-90). As mentioned, almost two dozen clinical trials have been rapidly developed that aim to exploit the toxin gene and bystander effects to achieve antitumor activity.

3.1.3. Other toxins

In addition to the *tk*/GCV system, several additional combinations of enzyme/prodrug have been developed to improve the efficacy of molecular chemotherapy (Table 5). Features of these combinations might overcome the limitations of *tk*/GCV. For example, some of them induce toxic effects not only in cycling but also in non-cycling cells (carboxypeptidase G2, nitroreductase, purine nucleoside phosphorylase). With others, the bystander effect is stronger (purine nucleoside phosphorylase) or does not require cell contact (cytosine deaminase, nitroreductase) (Table 5).

The combination of cytosine deaminase (CD) and 5-fluorocytosine (5-FC) has been the first of these alternative systems to be tested clinically (98). Studies *in vitro* and *in vivo* have shown that transfer of the microbial CD gene sensitizes cells to the innocuous antifungal drug 5-FC. This effect is induced by metabolizing 5-FC into the toxic antitumor agent, 5-fluorouracil (5-FU) (95). By administering high doses of the non toxic 5-FC, intratumoral activity of the enzyme can provide increased intratumoral concentrations of the active drug, without its accompanying systemic toxicity. Potential weaknesses of the system are its dependence on cellular proliferation, and its complex metabolism, which facilitates acquired resistance by tumor cells.

With some exceptions, single drugs in standard chemotherapy do not cure cancer. Historically, effective treatments were developed when drugs with different mechanisms of action were used in combination. Extending this concept to molecular chemotherapy, several combinations of

enzyme/prodrug have been shown to induce synergistic killing effects *in vitro* (110; 111). Combination schemas have achieved also higher rates of tumor regression and cure in animal models (112; 113). The application of classical principles for designing drug combinations would recommend the use of prodrug/enzymes that target both dividing and non-dividing cells, that elicit different mechanisms of bystander effect, and that have non-overlapping toxicities.

3.2. *Targeting*

Therapies based on gene transfer have been shown to be remarkably successful in *in vitro* and *in vivo* animal model systems. However, overriding limitations have consistently been made apparent in pre-clinical experiments and in the first human gene therapy clinical trials. Most of current difficulties in obtaining clinically relevant benefits have come from the insufficient efficiency of current gene vectors in transducing target organs, tumors or immune cells, and their inability to access in a selective manner target cells distributed systemically.

With molecular chemotherapy, the specificity of tumor eradication is based upon selective toxin expression in the neoplastic cells. Targeting in this context is critical for the reasons mentioned above. First, highly efficient transduction of tumor cells is needed to achieve therapeutic levels of toxin production. Second, transduction of normal cells should be avoided to reduce toxicity. Initially, these goals were attempted by anatomically directing the injection of gene transfer vectors to the site or compartment where tumor was located, and occasionally exploiting also the natural tropism of retroviral vectors for dividing cells. The diversification of available vectors, the continuing effort to develop vectors for systemic administration, and safety requirements, all led to the design of more precise targeting maneuvers. These are of two general types: either transductional targeting, whereby the toxin is specifically *delivered* to the tumor by means of a targeted gene delivery vector, or transcriptional targeting, whereby tumor or target tissue-specific transcriptional activators are employed to selectively *express* or 'turn on' the toxin gene exclusively within the tumor (Table 6).

The ability *to alter the binding tropism of viral vectors* is based on an understanding of the basic biology of viral entry. Modification of tropism involves altering initial binding to target cells via either genetic or immunological methods. In this regard, two distinct effects must be achieved: (i) ablation of the endogenous binding specificity, and (ii) preservation of the efficient, post-binding cellular entry mechanisms. Implicit in this strategy is the assumption that the virus can accomplish distal steps in

3.1. Toxin genes

3.1.1. Thymidine kinase

The most common molecular chemotherapy system utilized to date to accomplish cell killing has been the herpes simplex virus thymidine kinase HSV-*tk* gene given in combination with the prodrug ganciclovir (GCV) (74). The selectivity of the HSV-*tk* system is based on the fact that, contrary to normal mammalian thymidine kinase, HSV-*tk* preferentially monophosphorylates GCV, rendering it toxic to the cell. GCV is then further phosphorylated by cellular kinases to triphosphates that are incorporated into cellular DNA. The incorporation of the triphosphate form of GCV causes inhibition of DNA synthesis and of RNA polymerase, leading to cell death (69). Thus, tumor cells (or any other cell undergoing mitosis) transduced to express the viral *tk* gene have enhanced sensitivity to cell killing after exposure to GCV. Somewhat unexpectedly, normal cells transduced with HSV-*tk* after intravenous (75) or intrahepatic (76) administration of adenoviral HSV-*tk* vector have also shown high sensitivity to GCV, leading to liver degeneration and low survival in mice. The absence of toxicity of GCV after intravenous administration of a control adenovirus, or subcutaneous administration of an adenovirus encoding HSV-*tk*, suggests that the toxicity is specifically liver-associated. The relationship between toxicity and the proliferative status of liver parenchymal cells remains to be determined. Toxicity and efficacy of the transfer of HSV-*tk* is currently being tested in more than two dozen human clinical trials, including tumors of the ovary, brain, prostate, head and neck, mesothelioma, multiple myeloma, leukemia, and liver metastasis of colon cancer (for an updated list of protocols visit the Office of Recombinant DNA activities website at http://www.nih.gov/od/orda/protocol.htm).

3.1.2. Bystander effect

While the benefits of selectively eradicating tumor cells are obvious, an important limitation associated with molecular chemotherapy is the inability to target 100% of the tumor cells with the toxin gene. However, this may prove not to be as severe a limitation as initially believed due to the phenomenon known as 'bystander effect', whereby eradication of HSV-*tk* transduced cells elicits a killing effect upon the surrounding non-transduced tumor cells. That not all of the tumor cells need to contain the HSV-*tk* gene for obtaining complete eradication of the tumor was an observation of early experiments employing the relatively inefficient retroviral vectors in brain tumors (77; 78). This occurrence was later confirmed in a variety of other tumor model systems (79; 80). Our laboratory

its entry pathway after internalization via the heterologous cellular pathway. Sufficient understanding of both retroviral and adenoviral entry exists to allow the development of strategies to modify vector tropism. However, structural requirements during retroviral binding and entry have impeded successful exploitation of this knowledge (140). In contrast, the adenoviral particle has been more permissive for genetic and immunologically based changes towards tropism modification. Studies suggest that the requirements of this strategy may be met in the context of adenovirus-based vectors. This would allow the derivation of a vector with cell-specific gene delivery capacity and, in contrast to retrovirus, endowed also with *in vivo* stability. Such adenoviral vector would thus be suitable for application in the context of targeted gene delivery to disseminated diseases.

Although extensively tested (141), the use of transcriptional regulatory sequences for restricting the expression of therapeutic genes to the target tissue or tumor has shown inconsistent results in different vectors, with mounting evidence that *cis* elements located in the viral genome can alter both tissue specificity and activity of the promoter (142). Further limitations come from the prohibitively large size of regulatory sequences in the context of current vectors. Novel gene transfer systems with larger capacity, however, are being developed and can overcome this limitation, such as adenoviral "gutless" vectors (143), recombinant herpes virus (144), and human artificial chromosomes (145). For detailed reviews see general references on vector targeting for cancer gene therapy (146; 147; 148; 149) and on targeting of particular vector systems (140; 150).

3.3. *Drug-resistance genes*

In a second molecular chemotherapy approach, the host tolerance to higher doses of standard chemotherapeutic drugs is increased by transducing bone marrow cells, known to be highly sensitive to chemotoxicity, with genes that confer drug resistance (152; 153). In this context, retroviral vectors have been the vectors of choice for the *in vitro* derivation of stably transduced cells, due to their capacity for integration in cell chromosomes. It has been shown that when mice transplanted with bone marrow cells containing a transferred multiple drug resistance (MDR1) gene were treated with the cytotoxic drug taxol, a substantial enrichment for transduced bone marrow cells was observed. This demonstration of positive selection established the ability to amplify clones of transduced hematopoietic cells *in vivo* and suggested possible applications in human therapy. In a clinical trial, the bone marrow hematopoietic cells of refractory ovarian cancer patients will be transduced *ex vivo* with a retroviral vector encoding the MDR1 gene. After treatment for systemic disease with high

dose chemotherapy, the modified progenitor cells containing the MDR1 gene will be transplanted into the recipient and the patients will receive cyclic chemotherapy with taxol. Thus, it is expected that a treatment cycle-dependent enrichment of the marrow with hematopoietic cells resistant to the myeloablative effects of the chemotherapeutic drugs will be observed (154). Some potential problems with this strategy are, however, apparent. These include the failure to demonstrate that higher chemotherapy doses translate into improved patient survival, very low transduction efficiency of the target human hematopoietic cells with retrovirus vectors, the dose-limiting effects determined by other nonhematological toxicities, and the fact that cancer cells in the marrow could be transduced with the drug-resistance gene, which could rapidly give rise to clones of treatment-resistant tumor cells.

3.4. Chemosensitization and radiosensitization

A third approach of molecular chemotherapy seeks to modulate the level of expression of a variety of genes that influence the sensitivity of the cell to toxic stimuli, including conventional chemotherapeutic drugs and radiotherapy. Genetic chemosensitization can be achieved by modulating apoptosis, inhibiting tumor cell resistance, or enhancing intratumoral production of cytotoxic drugs. To facilitate apoptosis, genes such as *p53* may be administered to tumor cells to enhance the mechanisms of apoptosis induced by chemotherapeutic agents (155). Our group has shown that down-regulation of Bcl-2 protein levels by an intracellular anti-Bcl-2 single-chain antibody increases drug-induced cytotoxicity (156). Analogously, genetic downregulation of cellular factors related to chemoresistance has been shown to enhance chemosensitivity. Again, we have been able to show that single-chain antibody-mediated abrogation of the erbB-2 oncoprotein can significantly mitigate intrinsic chemoresistance in erbB-2 overexpressing ovarian cancer cells and allows for augmented sensitivity to the DNA-damaging drug cisplatin (37). Alternatively, genes can be administered intratumorally that enhance metabolic conversion of conventional chemotherapeutic agents. Studies have shown that transfer of a liver cytochrome P450 gene, CYP2B1, into human breast cancer cells greatly sensitized these cells to the cancer chemotherapeutic agent cyclophosphamide as a consequence of the acquired capacity for intratumoral drug activation. This effect produced a substantially enhanced antitumor activity *in vivo* (157). Lastly, combinations of conventional chemotherapeutic agents and molecular chemotherapy can serve the established rule of administering cytotoxic drugs with different mechanisms of action and toxicities. For example, one

clinical trial evaluates the association of adenovirus-mediated transduction of ovarian cancer cells with the *tk* gene followed by administration of acyclovir *and* the chemotherapeutic drug topotecan (http://www.nih.gov/od/orda/protocol.htm).

Several drugs are proven radiosensitizers, a fact that is commonly exploited in the clinic. One of these drugs is 5-fluorouracil (5-FU), which is the product of the CD suicide gene. In this regard, molecular chemotherapy based on CD has been shown to enhance the effects of radiation therapy in animal models of gliosarcoma (158) and cholangiocarcinoma (159). Thus, strategies to alter both chemosensitivity and radiosensitivity by gene transfer appear to have potentially wide applicability in many tumor contexts.

3.5. Obstacles to molecular chemotherapy

With all its promise, molecular chemotherapy also bears some practical limitations. To date, the strategy of molecular chemotherapy has been mainly used in loco-regional disease models. In these *in situ* schemas, a vector encoding the toxin gene is administered intratumorally or into an anatomic compartment containing the tumor mass. The goals of this delivery method are to achieve high local vector concentration in order to favor tumor transduction and to limit vector dissemination. However transduction efficiencies of presently available vectors have been shown to be inadequate. Even in closed compartment delivery contexts, it has not been possible to modify a sufficient number of tumor cells to achieve a relevant tumoral response in clinical models (160-164). Furthermore, although transduction with HSV-*TK* followed by ganciclovir treatment reduces tumor burden and prolongs survival in various model systems, including those utilizing intratumoral and intraperitoneal administration, the required increased doses of viral vector needed for obtaining quantitative tumor cell transduction is associated with limiting toxicity. In fact, substantial toxicity and experimental animal death has been noted (75; 163; 76) Thus, the small therapeutic index of currently available vectors in the context of *in situ* administration is a critical limiting factor for the purpose of gene therapy of cancer. Furthermore, and most importantly, a well-known limitation of conventional chemotherapy is also to be expected with the use of molecular chemotherapy, i.e. the appearance of drug-resistant tumor subpopulations (Table 1). In conclusion, vector limitations and well-known barriers to classical cytotoxic maneuvers impede the full exploitation of the promise of a more selective eradication of carcinoma cells via expression of toxin or protective genes.

4. Genetic Immunopotentiation

The development of clinically evident tumors implies the obvious failure of the host immune system to recognize and eliminate tumor antigen(s). Genetic immunopotentiation strategies attempt to achieve active immunization against tumor-associated antigens by gene transfer methodologies applied either to tumor cells or to cellular components of the immune system. To this end, recent insights into the pathophysiology of tumor escape from the immune system surveillance offer guidance for designing new therapeutic strategies (165) (Table 6).

In contrast to the evidence for tumor escape, there is another rare clinical observation that indicates the potential for an effective therapeutic maneuver against cancer based on the genetic modulation of the host immune system. This is the reproducible observation in clinical trials of immunotherapy against renal cancer and melanoma of dramatic spontaneous remissions of untreated patients with bulky, solid metastatic cancer, in a measurable proportion of cases (166). This unexplained observation suggest that the immune system can occasionally recognize and reject large volumes of tumor, and supports the undertaking of ambitious approaches for genetic immunopotentiation against cancer.

4.1. Tumor escape

Factors that can explain the failure of the immune system in the cancer patient are an inadequate immunogenicity of the tumor or a deficiency of the immune system to 'recognize, respond and reject'. Reduced tumor immunogenicity can be related to the absence of either tumor-specific antigens or MHC I molecules on the tumor cells, which are essential for presentation of cellular antigens to effector $CD8^+$ T lymphocytes. Alternatively, it may well be that the lack of costimulatory molecules, such as B7, in tumor cells establishes immune tolerance or ignorance, which keeps the tumor from being treated as foreign or dangerous. Immune system deficiencies, in turn, can be either generalized or regional, including in the last case the active suppression by the tumor of host antigen presenting and effector cells in the local microenvironment.

4.1.1. Tumor antigens

Tumor cells have been shown to express *tumor antigens*, i.e. cell determinants that can be recognized as extraneous by the immune system, at least in *in vitro* and syngeneic animal studies. Protective

immunity in early animal studies was shown to depend on $CD8^+$ cytolytic T lymphocytes (CTL). To be recognized by T lymphocytes, tumor antigens should be intracellularly associated with MHC class I or class II molecules and then presented to cells of the immune system on the tumor cell surface. Some clones of tumor infiltrating lymphocytes (TIL) can lyse the majority of cancer cell lines of certain types, such as melanoma HLA-A2+, which suggests that there are antigens shared by tumors of the same histology from different individuals. It has been argued that this fact supports the development of universal vaccines against certain tumors.

There are five general *categories of tumor antigens.* First, antigens coded by genes that are silent in normal cells and expressed almost uniquely in tumors, such as the MAGE family of antigens in melanomas. Second, antigens resulting from mutations in normal proteins, such as p53, MUC-1, MUM-1, CDK4, and beta-catenin. Third, differentiation antigens, present normally in the tissue from which the tumor originates, such as MART-1, gp75, gp100, and tyrosinase, again in melanoma and melanocytes. Fourth, antigens encoded by normal genes that are dysregulated in tumor tissues, such as erbB-2. Five, antigens encoded by genomes of oncogenic viruses, such as the human papillomavirus E6 and E7 gene products. Notably, the role of CTL against these antigens in the rejection of human tumors has not been demonstrated in any case.

To identify tumor-associated antigens, mice have been immunized with tumor cells, and the resultant monoclonal antibodies have been used to isolate the corresponding peptides from the surface of human melanoma cells. Due to the methodology used, which identifies any cognate antibody ligands, many of these antigens are not related with the neoplastic phenotype and are not necessarily immunogenic in humans. As mentioned, the activation of T lymphocytes, i.e. the cellular immune response, is more important than the humoral response for inducing tumor lysis and regression in experimental models. Therefore, the search for relevant antigens is currently based in the analysis of reactive circulating or tumor-infiltrating T lymphocytes isolated from patients with tumors (167; 168). More than a dozen specific peptides recognizable by CTL have been identified to date. Unfortunately, after vaccination of melanoma patients with these peptides only sporadic responses have been observed. More importantly, in cases showing tumor regression, it has not been possible to correlate response with *in vitro* CTL responses. The existence of tumor-specific antigens, it appears, does not assure the development of an effective immune response against tumors.

A *theoretical barrier* for antitumor vaccination, probably related to the observation just mentioned, can be deduced from the initial experiments done with tumor transplants in mice. It was observed that some tumors induced by carcinogenic agents were clearly immunogenic when transplanted into basically identical syngeneic animals. Paradoxically, primary tumors were not rejected in the original donor animals. In addition, lethally irradiated tumor cells were still capable of immunizing naïve animals against later exposure to tumorigenic doses of the cells. However, late vaccination of a tumor-bearing host could lead to rejection of a second fresh tumor cell challenge, but the original tumor was usually not rejected. Conceivably, tumor antigens can be recognized, in the murine model, only after previous exposure or a change in the circumstances of exposure of the immune system to the tumor cells. Thus, the mere presence of antigens in tumors may be not enough for inducing an efficient antitumor immune response in the natural tumor site, which probably involves the attraction and activation of tumor-specific T cells at the site of established tumors.

4.1.2. Major Histocompatibility Complex

MHC class I products, strongly expressed on normal tissues, are occasionally absent or down-regulated in tumors of varying histology, in comparison with their respective normal counterpart tissues. Sometimes this down-regulation occurs more significantly in metastatic lesions, or in later steps in tumor progression (169). Supporting the potential role of changes in MHC levels in tumor escape, it has been shown in co-cultures of cancer cells and $CD8^+$ lymphocytes that cancer cell clones can arise that 'escape' from lysis by autologous lymphocytes (170; 171), and that this phenomenon correlates with a marked decrease in the expression of MHC class I molecules. By itself, this observation could explain the local failure of the immune system to develop a strong antitumoral response in the clinical context. Thus, provision of MHC I expression in previously negative tumor cells could restore locally the functionality of the cytotoxic cellular immune response. In this regard, is also interesting that gamma interferon and tumor necrosis factor induce in several tumor cell lines an increase in the expression of MHC class I and, under experimental conditions, can restore the antitumor lytic functions of the cytotoxic cellular immune response (172).

The molecular basis of tumor escape has been further documented in a fascinating clinical case. A recurrent lesion was studied six years after resection of the primary tumor, a malignant melanoma. The new lesion had concomitantly lost both a tumor-associated antigen and the protein TAP-1. This protein normally carries peptides from the cytoplasm to the endoplasmic reticulum, where they are

associated with MHC class I molecules previous to their transport to the cell surface. Transfer of genes encoding the lost antigen and TAP-1 into tumor cells grown from the recurrent lesion restored sensitivity of the tumor cells to CTL (173). Thus, tumor-associated antigen expression and presentation may be not sufficient, but is clearly needed for development of an efficient cellular immune response.

4.1.3. Costimulatory signals and antigen presenting cells

In effect, rejection of the tumor by CTLs requires not only the presence of tumor antigens and their appropriate display in association with MHC class I molecules (signal 1) but also an array of *costimulatory signals*, provided by antigen-presenting cells (APCs) (signal 2). These cells, derived from the bone marrow, are critical for the activation of CTL, which happens through the interaction of molecules of the B7 family, present on APCs, and the CD28 receptor on T cells. This interaction occurs in the lymph nodes and requires the previous activation of the APC in an inflammatory environment (174). Once activated, T cells expand and are then able to migrate, seek, and destroy tumor cells that express the appropriate antigen in a MHC context. A critical observation for understanding tumor immunology and tumor escape is that the interaction of CTL and tumor cells in the absence of activated APCs results in a state of antigen-specific T cell dormancy referred to as anergy (175). The molecular basis of this phenomenon is unclear. In this regard, it has been speculated that a factor secreted by the tumor cell alters the lymphocyte TCR-CD3 surface complex, resulting in impaired intracellular signal transduction (176). Thus, several ingredients of an effective immune response may be absent in the relationship between the tumor and cells of the immune system in normal conditions, favoring the development of weak and ineffectual responses and lack of tumor eradication.

4.1.4. Immune system exhaustion

The simplest explanation for tumor escape is that the rapid growth and spread of the tumor overwhelms the effector mechanisms of immune responses. However, there is evidence against the exhaustion hypothesis as the explanation for the growth of cancer cells in immunocompetent animals. Elegant experiments in T cell receptor transgenic mice have shown that, even when essentially all T cells recognize a given antigen, *tumors* expressing the antigen are not rejected whereas *skin* expressing the same antigen does. Therefore, systemic T cell exhaustion or antigen-specific

anergy is not responsible for the growth of the antigenic cancer cells, at least in this experimental model (177). Incidentally, is clear in addition that factors at the site of established tumors inhibit the immune response in a strictly local manner.

4.1.5. Immunosuppression

The immune system deficiency present in the tumor microenvironment may be related to the local production of immunosuppressive gene products by tumor cells or infiltrating leukocytes. Candidate molecules thought to mediate this local immunosuppression are transforming growth factor beta (TGF-ß), interleukin-10, vascular endothelial growth factor (VEGF), and Fas ligand.

TGF-ß is secreted by a variety of malignant tumors and their supporting stroma. In addition to other effects on cellular proliferation and the extracellular matrix, TGF-ß elicits potent growth inhibitory effects on B and T lymphocytes; it suppresses the generation of cytotoxic T and NK cells, down-regulates high affinity IL-2 receptors, and blocks the synthesis of cytokines in peripheral T-lymphocytes. Tumor cells induced experimentally to produce TGF-ß, though retaining expression of MHC class I molecules and tumor-specific antigens, do not stimulate primary CTL responses *in vitro* and are not effective *in vivo* for directly stimulating primary CTL or in priming for CTL responses (178). Furthermore, TGF-ß-producing tumors grow progressively in transiently immunosuppressed mice without losing the tumor antigen. More direct evidence has been obtained by using antisense molecules against TGF-ß. Rats that were implanted with glioma tumor cells transfected with an antisense plasmid survived significantly longer than controls, and had effector cells in lymph nodes with increased lytic activity, as determined by *in vitro* cytotoxicity assays (32). Thus, TGF-ß produced by tumors may promote escape from immune surveillance through local immunosuppression.

Interleukin-10 (IL-10) has potent anti-inflammatory and immunosuppressive properties. It blocks the release of mediators of inflammation by macrophages, and reduces the presentation of costimulatory molecules by APCs and tumor cells to lymphocytes. Its presence has been detected in several tumors, and especially high serum levels have been detected in advanced tumor stages (165). One common clinical observation in the treatment of disseminated cancer is the diverse response of different metastatic lesions to a single cycle of antitumoral treatment. Recently, the function of APCs in regressing and non-regressing lesions has been compared. APC from regressing lesions were significantly more potent inducers of allogeneic T lymphocytes than APC from progressing

lesions. Furthermore, APCs from progressing lesions were able to induce anergy *in vitro* in $CD4^+$ lymphocytes. IL-10 could be detected in the culture media of cells isolated from progressing lesions, but not in the culture media of cells from regressing lesions, where the stimulating cytokines IL-2, interferon-gamma, and IL-12 were detected. These results show that mediators produced in some metastatic foci induce a change in APC function whereby tumor tolerance, instead of costimulation against the tumor, is induced, thus silencing the antitumoral immune response (179).

Recently, human cancer cell lines have been shown to release a soluble factor(s) that very remarkably inhibits maturation of precursors of dendritic cells (DC), one kind of APC, without affecting the function of relatively mature DCs (180). This local immunosuppressive activity was determined to be, at least in large part, due to VEGF. Thus, VEGF may have a broader role in tumor development than its fundamental role in angiogenesis would suggest, with interference in maturation of DCs being a novel mechanism of tumor promotion of this vascular growth factor.

An additional mechanism of 'tumor escape' from the immune system is the expression by tumor cells of Fas ligand (FasL). When FasL on tumor surface binds to Fas, present in the cellular membrane of cytotoxic T lymphocytes, a series of events is triggered in the lymphocyte by Fas that lead to apoptosis (181). Thus, the expression of FasL by tumors may protect them against T cell-mediated immune rejection. For instance, this mechanism has been demonstrated to be operating in all samples analyzed from seven patients with melanoma, as shown by the presence of FasL in the tumors and concomitant evidence of apoptosis in tumor infiltrating lymphocytes (182). The presence of immunosuppressive factors such as FasL and others in tumors clearly suggests the need to complement *any* immunotherapy strategy with maneuvers explicitly addressing the intratumoral presence of inhibitors of the immune system response.

4.2. Genetic modification of immune effector cells

The growing knowledge on tumor immunobiology has guided the development of strategies for genetic immunotherapy against cancer (167). Two types of general interventions have been applied to increase the ability of the patient to mount an efficient antitumor immune response. First, cells of the immune system have been modified to augment their capacity for recognizing and rejecting tumor antigens. Second, the tumor cells themselves can be altered to increase their immunogenicity. Gene therapy offers the possibility of genetically modifying both types of cells, and, importantly, this

intervention can be restricted regionally, thus avoiding the intolerable toxicity that characterizes most biologic response modifiers when administered systemically.

4.2.1. Tumor infiltrating lymphocytes (TIL)

Several therapeutic maneuvers have been based in trying to modify the cells of the immune system. TILs are derived from mononuclear cells obtained from leukocytes infiltrating resected specimens of solid tumors. In the early 1990s, it was hypothesized that TILs could be an enriched source of NK cells and CTLs specific for tumor antigens, and could also have tropism towards systemic tumor foci. On this basis, technology for their expansion in culture was developed and TILs were the first immune cells to be genetically modified and applied in a human gene therapy clinical trial against cancer (183). It was soon observed that while TILs do include CTL and NK activated cells, only a few of these cells in these mixed populations are specific against the tumor from which they are isolated. Furthermore, reinfused TILs localized poorly into tumors.

Three strategies have been applied to improve treatments based on TILs (184). First, the IL-2 gene has been transferred into TILs to increase lymphocyte number and survival when in the host. Second, to boost antitumoral efficacy the gene of tumor necrosis factor (TNF) has been transferred into TILs *ex vivo* previous to reinfusion. Thirdly, to improve localization into tumor foci several novel cellular receptors have been engineered in lymphocytes by genetic and immunological means. In this regard, Steven Rosenberg, who pioneered clinical trials with TILs at the National Cancer Institute, has reported rare clinical responses, with notable toxicity from the IL-2 treatment frequently added to maintain the requisite number and functionality of TILs. Localization of TILs in tumor biopsies is, as mentioned, modest. Targeted lymphocytes expressing chimeric T-cell receptors against tumor antigens have been developed with the aim of improving that barely exploitable tropism, and are currently undergoing clinical testing in colorectal and ovarian cancer, and melanoma.

4.3. Genetic modification of tumor cells

An alternative strategy for trying to augment the antitumor immune response is to genetically modify tumor cells, or to manipulate their components, to facilitate the start of a robust immune response. Thus, it has been hypothesized that a formerly tolerant host may revert its immune status, characterize by tolerance or anergy, and ultimately experience tumor rejection. In other words, it is

hypothesized that the host can be 'vaccinated' against the tumor by exposing tumor antigens to the immune system in a more favorable context (185; 186; 187). Most clinical experience with antitumor vaccines to date has been obtained in melanoma patients. For years, irradiated tumor cells, either autologous or allogeneic, have been administered in combination with different adjuvants, such as BCG. Later, the molecular definition of tumor-associated antigens allowed the testing of vaccines based on individual antigenic determinants delivered to the patient in the form of peptides or DNA. More recently, tumor cells themselves have been genetically modified to increase their immunogenicity by transfer of a variety of genes, including cytokines such as GM-CSF, costimulating molecules such as B7, and MHC molecules. A common requirement, not adequately accomplished routinely yet, is to introduce the gene of interest in tumor explants or cultured cells with high efficiency.

4.3.1. Cytokine gene transfer

The utility of antitumor vaccines based on whole tumor cells has been poor when analyzed in randomized clinical trials. However, it has been argued that the genetic modification of tumor cells with cytokine genes could augment the immune response against the tumor (188). In fact, this maneuver could overcome the defects mentioned above which are related to escape of the tumor from immune surveillance. One possible intervention is to induce, by intratumoral gene transfer, the secretion of cytokines that, once secreted by the tumor cell, can activate directly the response of CTLs, and increase the awareness of the immune system cells to tumor cells in the tumor microenvironment. Cytokines tested to increase the antitumor immune response include: IL-1, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IFN-γ, TNF, G-CSF and GM-CSF. In some cases, intense inflammatory infiltrates have been observed surrounding cytokine-secreting tumor cells, with the type of infiltrate varying with the particular cytokine. In animal models, immune-mediated tumor regressions are consistently observed with this strategy. Also, specific immunity mediated by CTLs against subsequent exposures to unmodified tumor cells is commonly observed in treated animals. More than twenty clinical trials are evaluating the effect of treatment with autologous or allogeneic tumor cells modified by cytokine genes. Preliminary results, reported in a trial using IL-2-secreting tumor cells, reveal poor tumor responses and a low frequency of CTLs specific against the tumor (189). If confirmed in other trials, these results should perhaps not be surprising, given the

recognized complexity of immunological phenomena and the one-sidedness of the single-gene interventions being tested.

4.3.2. Induction of MHC expression and transfer of allogeneic MHC

Recognition of a tumor-associated antigen by CTLs requires its simultaneous presentation with MHC class I molecules on the surface of tumor cells. As mentioned, there is evidence for a decrease in MHC class I levels in numerous tumors. Interferon-γ stimulates the expression of MHC molecules, and can therefore make tumor cells more recognizable by the immune system. Studies *in vitro* have confirmed that transfer of the interferon-γ gene increases MHC expression, and these results are the foundation for currently ongoing clinical trials (192). Interestingly, tumor transfer of allogeneic MHC genes can generate CTLs reactive not only against the treated tumor mass, but also against non-modified tumor cells, and results in widespread tumor regression (193). With this basis, clinical trials have been performed by transferring the gene of HLA-B7 by intratumoral injection into tumors that do not express the molecule (194; 195). A theoretical risk of this strategy is the inhibition of the antitumor activity of NK cells, which recognize and attack specifically MHC class I-negative cells.

4.3.3. Costimulatory molecules

Costimulatory signals promote clonal expansion of antigen-specific T cells and their differentiation into effector and memory cells. The efficacy of increased costimulation of T lymphocytes for antitumor immunotherapy has been shown in mice vaccinated with tumor cells expressing genes encoding several costimulatory molecules. For instance, tumor cells transfected with B7-1 (also known as CD80, a ligand of CD28 in lymphocytes and distinct from HLA-B7) potently stimulate an immune response, which is not observed with unmodified cells. When injected into syngeneic animals, B7-1 expressing cells are rejected, whereas unmodified cells are not. Furthermore, B7-1 expressing cells induce a potent immune response against unmodified cells in distant regions. The reason is probably that the presence of B7-1 increases the activation of T lymphocytes, but the cytolytic activity of differentiated CTLs does not depend on B7-1. These results in animals led to the development of clinical trials, in which tumors from patients are cultured, genetically modified, irradiated, and finally reintroduced into the same patient as vaccines. This strategy, although time-consuming, technically demanding, and costly, could be effective even when no tumor antigens have

been identified. Experiments mentioned above, however, should be remembered. In immunized mice, an immune response could be observed against freshly injected subcutaneous tumors, whereas no response was observed against concomitant primary, established tumors, and that this was due to a lack of mobilization of activated T lymphocytes towards the original tumor localization. Immune ignorance of primary tumor sites can therefore severely limit the utility of strategies based on costimulatory molecules.

Another important couple of accessory molecules is formed by the intercellular adhesion molecules ICAM-1, ICAM-2, ICAM-3 and the lymphocyte functional antigen (LFA-1). Many tumors do not express ligands of the ICAM family efficiently, thus decreasing their ability to costimulate or be targets of the immune response. Again, the transfer of the corresponding gene could conceivably be useful to augment the effect of antitumor vaccines, and that seems to have been the case in several animal models (196; 197).

CD40, present in APCs, and its ligand in lymphocytes, CD40L, form a third system of costimulatory molecules of unclear importance with respect to the systems mentioned above.

4.3.4. Vaccination with tumor-associated antigens

Antitumor vaccines have also been developed based on tumor-associated antigens. Using TILs able to induce regression of tumors as a source, genes encoding the cognate tumor antigens have been isolated and cloned (198). Patients could be vaccinated with these tumor-associated antigens combined with adjuvants that increase their immunogenicity. The rationale is the probability that existing T lymphocytes with antitumor specificity can be activated and specifically stimulated by the vaccine. Immunization could then be done by direct administration of the peptides, by viral vectors encoding the antigens, by cellular lysates obtained after infection of tumor specimens with vaccinia vectors encoding the antigens, or by naked DNA.

One limitation of vaccines based on peptides is that it is improbable that these peptides could efficiently replace other non-immunogenic peptides already present and associated with MHC molecules in the surface of tumor cells in the patient. Furthermore, extracellular proteins are presented to the immune system via MHC class II molecules, which activate $CD4^+$ helper lymphocytes but not cytotoxic $CD8^+$ lymphocytes. In contrast, DNA vaccines are better stimulators of CTL by leading to expression of antigens from within cells, where they are associated with MHC

class I molecules and presented in the appropriate manner in the cell surface. Particularly attractive candidate antigens especially are those shared by multiple tumors, such as the melanoma antigens MAGE-1, MART-1 y gp100, which are currently being used in several ongoing clinical trials.

4.4. *Obstacles to genetic immunopotentiation*

The main advantage of genetic immunopotentiation is the possibility of enlisting physiological mechanisms for a potentially vast amplification of the therapeutic maneuver. To this end, even modest levels of gene transfer can potentially be followed by clonal expansion and systemic spread of effector immune cells and mediators. Thus, efficiency of gene transfer here is not critical, given the relatively low amounts of cells and gene products needed to obtain a potentially powerful response from the immune system. There are, however, other more important obstacles that perhaps explain the *poor* results obtained to date by tumor immunotherapy in humans. Antigenic heterogeneity and plasticity, redundancy of immune system regulation, and well-established tolerance to natural tumors are the more evident barriers.

4.4.1. Antigenic heterogeneity and plasticity

During the last two decades numerous reports have confirmed both *in vitro* and *in vivo* that expression of tumor cellular antigens in different tumor types is heterogeneous, with variability being found not only between different patients with the same tumors, but between different regions of a single tumor and even in single cell clones (199; 200) Moreover, this variability changes with time. This fact, first confronted by monoclonal antibody therapists, may clearly limit the impact of vaccines against single tumor-associated antigens, and even those based on cultured, homogeneous populations of tumor cells.

4.4.2. Redundant phenomenology of the immune system

The destructive power of the immune system, occasionally needed in its entire exuberance, obligingly requires a complex network of balances and counterbalances to control the pathways of activation and termination of the immune response. Interventions directed to supplement or inhibit single mediators will most probably obtain partial physiological and therapeutic results in the best case, may frequently yield no result at all, and occasionally will produce effects opposed to those desired. Increasingly, combinations of cytokines are being used to try to control the complexity of

the immune response against tumors. Current therapeutic interventions for inducing organ graft tolerance successfully prolong organ survival by blocking multiple effector cells and mediators of the adaptive and innate immune systems. Similarly, it is conceivably that breaking the tumor tolerance will require a strategy of multiple interventions including several target cells and cytokines.

4.4.3. Lack of immune response (tumor tolerance)

Current knowledge in tumor immunobiology establishes that T cells able to recognize tumor-associated antigens can be found *in vivo* and are inducible (with some difficulties) *in vitro*. Thus, the lymphocyte repertoire against these epitopes has not been deleted. However, either tolerance to these (tumor) self-antigens has been induced or, in the absence of costimulatory signals, peripheral T cells simply have ignored these antigens (201). This phenomenon should obviously be an early event in tumor progression (202), and may be totally missed in most animal models employed to date, which are based on tumor *grafts*. In this regard, studies with transgenic mice that develop *spontaneous* tumors have shown that vaccination with tumor cells transduced with cytokines fail to inhibit tumor onset and progression, whereas the same cells are able to immunize non-transgenic mice subsequently grafted with tumors (203). Thus, the failure of naturally established tumors to efficiently present antigens, and to attract and activate tumor-specific T cells at the tumor site may impede successful vaccination against cancer antigens. Furthermore, ignorance by the immune system can abort most of the immunotherapy maneuvers being tested and discussed above.

5. Novel Strategies to Overcome Current Limitations

As we have reviewed above, gene transfer therapies are remarkably successful in *in vitro* and *in vivo* animal model systems. In effect, we already know that the malignant phenotype can be reverted in tumor cell lines by 'knocking-out' or adding certain genes; that tumors can be eradicated by delivery of cytotoxic genes followed by treatment with appropriate prodrugs; and that tumors can be cured in murine models by making the tumor cells either more immunogenic or by making the immune system cells more responsive, via the expression of cytokines, or costimulatory and immunogenic molecules. However, overriding limitations have been made apparent in preclinical experiments and in the first human gene therapy clinical trials against cancer, as emphasized by the Orkin and Motulsky Report to NIH (204). Most difficulties in obtaining clinically relevant benefits come from the inefficiency of current gene vectors in transducing tumor or immune cells and their inability to

access in a selective way target cells distributed systemically. In this regard, each of these limitations specially undermines the implementation of one particular gene therapy strategy. Several avenues for improvement have been proposed, and some will be succinctly reviewed in this section.

5.1. *Mutation compensation requires quantitative gene transfer*

For mutation compensation strategies to work successfully, it seems that *every* tumor cell would have to be corrected in its genetic defect to achieve a therapeutic outcome. Thus, quantitative transduction of therapeutic genes into the tumor after *in situ* administration of the gene therapy vector is an essential requirement. To this end, a variety of vector amplification strategies are being explored, including replicative (205) and integrative viral systems (206).

5.1.1. Replicative vector systems

One method to circumvent suboptimal tumor transduction of therapeutic genes *in vivo* would be the use of conditionally replicative viral vectors. In this context, a replication-competent virus would be employed to replicate selectively within transduced tumor cells, leaving normal tissues unaffected. Production of progeny virions from the transduced tumor cells would then allow infection of neighboring cells. Thus, the intratumoral viral inoculum would increase, improving the tumor transduction efficiency. In addition, the use of viruses that display a lytic life cycle would allow virus-mediated oncolysis. This effect would occur irrespective of the delivered transgene. In either case, an amplification of the antitumor effect would be achieved (205; 207).

For *in vivo* models of this strategy, a virus with *in vivo* stability and the capacity for conditional replication within tumor cells is mandated. In this regard, recombinant adenoviruses and herpesviruses have the potential to provide the required properties. Not only do they display high efficiency and stability *in vivo*, but also their replication can be controlled. In the case of adenoviruses, adenoviral replication can be restricted to tumor cells by placement of genes needed for viral replication under the control of tumor or tissue-specific transcriptional control elements, such as the promoter of the prostate-specific antigen (PSA) for use in prostate cancer (208). Alternatively, mutant adenoviruses have been developed that exhibit selective replication in cells lacking functional *p53*. Because *p53* is absent in many tumors, a selectively replicative system based on this lytic virus has been proposed for cancer therapy purposes (120). However, extensive studies in a variety of cell lines and animal tumor models have to date failed to confirm the selective

properties of the virus to replicate only in *p53* mutant tumor cells. Further refinements in these replicative vectors are anticipated. For example, our group is developing defective adenoviral vectors that replicate selectively under the stimulus of the cytokine interleukin-6, or under the controlled addition of second vectors carrying replication-enabling DNA sequences (209; 210).

Herpesviruses have also been developed that replicate conditionally in dividing or tumor cells. This selectivity is based on several possible mutations engineered in the viral genome that prevent it from replicating unless the infected cell provides for a substituting molecular activity (211). These properties have established brain tumors, which are surrounded by non-mitotic cells, as an ideal therapeutic model for testing replication-conditional herpes vectors. Notably, human clinical trials have already begun to test both adenovirus and herpesvirus-based replicative vector systems.

5.1.2. Prolonged transgene expression: integrative vector systems

Lack of stability *in vivo* has confined the use of retroviruses to the *ex vivo* modification of target cells. For *in situ* gene delivery, vectors with high efficiency and stability *in vivo* are needed. Of vectors with both characteristics, adenoviruses have been most extensively characterized and used (Table 7). In addition to a significant inflammatory and immune response, an additional basis for the limited transgene expression associated with adenoviral vectors derives from their non-integrative nature, such that vector sequences are not retained in the host genome and are not inherited by progeny cells. In this regard, after adenoviral-mediated gene transfer, the recombinant genome is present epi-chromosomally in target cells. Thus, with the proliferation of transduced cells, vector sequences are lost, with the consequence of limited duration of transgene expression. For utility in mutation compensation, and in other gene therapy strategies, it thus would be desirable to develop methods to achieve integration of adenoviral vector-delivered transgene sequences in vector infected cells. As a novel approach to meet this need, we have develop a chimeric viral vector system that exploits favorable aspects of both adenoviral and retroviral vectors. In this schema, adenoviral vectors induce target cells to function as transient retroviral producer cells *in vivo*. The progeny retroviral vector particles can then effectively achieve stable transduction of neighboring cells (206). Thus, the principle of combining selected features of available vectors into novel chimeric vectors is already governing the development of virus-based gene transfer systems (212).

Lentiviruses are retroviruses that, in contrast to other members of the family, can infect both dividing and non-dividing cells. This fundamental feature has driven significant efforts for its development, although practical issues related to production and safety have limited its widespread use. Efficiency of transduction of most potential cellular targets by lentiviral vectors and *in vivo* utility have just begun to be described (213).

5.1.3. Prolonged transgene expression: immune tolerance to viral vectors

Gene delivery via adenoviral vectors has been associated with the induction of characteristically intense inflammatory and immunological responses when employed *in vivo*. A number of specific cellular and humoral immune effector mechanisms, together with nonspecific defense mechanisms, eliminate the infecting virus (214-216). This process, refined over the course of millennia for maximal efficiency, has been associated with attenuation of expression of the transferred therapeutic gene based, at least in part, on loss of the vector transduced cells. Based on an understanding of the biology of this phenomenon, specific strategies have been developed to mitigate this process (217).

Maneuvers to minimize the immune response against viral vectors include manipulations of both the vector and the host. Firstly, recombinant viral *vectors are genetically engineered* to *delete* viral genes encoding highly immunogenic or cytotoxic viral proteins. New generations of deleted viral vectors are, however, more difficult to propagate and still hardly devoid of immunogenic properties. Alternatively, different serotypes and adenoviruses of other species have been proposed to minimize the stimulus for an immune response. Secondly, vectors have been modified to *express* immunomodulatory molecules. It has been hypothesized that this could create a locally privileged environment for the vector. Some of these engineered molecules are viral genes that interfere with the apparatus of antigen presentation, such as the adenoviral glycoprotein 19K or the herpes simplex virus (HSV) immediate early protein ICP47. Others are recombinant molecules designed to abrogate antigen presentation, such as antisense oligonucleotides or single-chain antibodies against MHC class I and II proteins, or to block costimulation, such as CTL4IgG (217).

Interventions *on the immune system of the host* have been adopted from common practices in the field of organ transplantation. In this regard, virally transduced cells have been considered to behave, to some extent, as allogeneic cell transplants. Thus, drugs are employed that inhibit the cellular immune response, such as anti-CD4 antibodies, cyclosporine, dexamethasone, and FK 506. In addition,

drugs that decrease the humoral immune response, such as cyclophosphamide and deoxyspergualin, have been used. Recently, several groups have shown transient and more specific immune blockade with inhibitors of T cell costimulation, such as anti-CD40 ligand, CTL4IgG, and anti-LFA-1. Unfortunately, the required chronic administration of these immunosuppressive drugs affect systemic immune function and results potentially in a number of complications, such as infection and malignancy. This makes them less attractive in principle for clinical application, although short-term treatment in cancer patients should be feasible. Lastly, induction of tolerance to adenovirus vectors by oral ingestion of adenoviral antigens has been described, but this approach needs further characterization. Thus, although inflammatory and immunological issues have limited the overall utility of adenoviral vectors for gene therapy applications, many of the mentioned strategies appear promising and may ultimately allow overcoming these limitations.

5.2. *Molecular chemotherapy requires targeting*

In molecular chemotherapy strategies, other problems take precedence. Less than optimal transduction levels may require the employment of higher magnitudes of gene vectors leading to target cell cytotoxicity. Thus, for direct *in situ* infection of selected organs, improvements in basic gene transfer efficiency may be required. In addition, and more specifically, the promiscuous tropism of the vector may potentially allow ectopic transduction of non-tumor cells with toxic genes. Therefore, strategies to enhance the efficiency of the vector as well as methods to enhance the specificity of target cell transduction would be necessary to render gene delivery optimal for gene therapy purposes. In this regard, studies have demonstrated the feasibility of creating tropism-modified retroviral and adenoviral vectors to achieve cell-specific targeting, as described above. To this end, immunological and genetic strategies for retargeting vectors to non-viral specific cellular receptors have been designed. Modifications of the adenovirus vector to alter native viral tropism in order to achieve selective transduction of target disease cells have proved to be feasible (127; 218-220), in contrast with the more structurally demanding characteristics of retroviruses. Notably, targeting maneuvers have shown in selected cases the additional ability to *enhance* the transduction efficiency of the recombinant adenoviral vector by orders of magnitude, by binding and entering into the cell via heterologous cellular receptor pathways (unpublished).

5.2.1. Immunological targeting

To test our immunological schema of adenoviral targeting, we chose to target the folate receptor, which is overexpressed on the surface of a variety of malignant cells, including ovarian carcinoma cells. We conjugated the Fab fragment of an antibody against the adenoviral fiber with a folate moiety. When this Fab-folate conjugate was complexed with an adenoviral vector carrying a reporter gene, we observed redirection of the adenoviral vector via the folate receptor at high efficiency. Furthermore, when complexed with an adenoviral vector carrying the HSV-*tk* gene, we obtained specific killing of cells overexpressing the folate receptor (127). Thus, retargeting of adenovirus by bispecific conjugates based on anti-adenovirus antibody fragments and cellular receptor-specific cognate ligands was shown to be feasible, and similar strategies have been developed by other researchers (219). Importantly, this flexible strategy permits the rapid derivation and testing of targeted adenoviral vectors. In this regard, we have recently shown that recombinant adenovirus can be targeted specifically to a variety of cell types, including ovarian cancer cell lines, by exploiting the heterologous cellular pathway of basic fibroblast growth factor receptor (FGF-R). Here, the levels of gene transduction by retargeted adenovirus were even greater than those achievable by adenovirus alone. Therefore, an adenoviral vector targeted to the FGF-R would allow higher or at least similar levels of gene delivery than non-targeted virus and lower non-specific toxicity. This suggests that the small therapeutic index of currently available vectors in the context of *in situ* administration could be improved using targeted adenovirus for the purpose of gene therapy of cancer. In fact, recent experiments in our laboratory have confirmed that Fab-FGF retargeted adenovirus achieved enhanced *in vivo* expression of a reporter gene in an intraperitoneal tumor model of ovarian carcinoma in nude mice (128).

5.2.2. Genetic targeting

Strategies to derive a tropism-modified recombinant virus, most advanced with retroviruses and adenoviruses, have been directed towards modification of viral surface proteins to accomplish incorporation of heterologous cell-binding ligands. This approach thus capitalized on the extensive knowledge on the endogenous cell-binding ligands of both viral vectors. As with immunological strategies, retrovirus particles have been generally less tolerant to binding modification maneuvers. In contrast, with adenovirus several groups have been able to localize novel ligands in the cell-binding knob portion of the viral fiber (125; 126; 221) and in the penton base protein. Engineering

adenoviruses has thus improved its transduction efficiency in cells otherwise refractory to adenoviral gene transfer, such as endothelium and leukocytes. The achievement of these goals, even in a limited context, predicts that further analysis could identify, for particular target tissues, optimal ligands from the standpoints of cell binding and internalization. The possibility of employing therapeutic adenovirus vectors to selectively transduce cells in the context of disseminated disease is thus clearly open.

5.2.3. Definition of new targets

Ideally, new vectors will be administered by intravenous injection and will effect gene transfer specifically in systemically distributed target cells. Genetic and immunological targeting strategies allow for consideration of such a targetable, injectable vector, which is considered the "Holy Grail" of gene transfer. A systematic method for identification of ligands that can be incorporated into vectors for selective delivery to target cells is therefore in order. In addition to traditional ways of identifying single cellular receptors one-by-one, a high-throughput method has been developed based on the technology of phage display libraries. In this approach, tens of millions of short peptides can be rapidly surveyed for tight binding to a cellular receptor using an "epitope library." The library is a vast mixture of filamentous phage clones, each displaying one peptide sequence on the virion surface. The survey, originally performed by using the binding protein to affinity-purify phage that display tight-binding peptides (222), can also be accomplished *in vivo* by propagating isolated phage from target organs after intravenous injection of the phage random library (223). After propagation of isolated phage in Escherichia coli, the amino acid sequences of the peptides displayed on the phage are then determined by sequencing the corresponding coding region in the phage DNAs. Thus, peptides with selective tropism for target organs can be isolated and ultimately used as binding motifs in engineered viral vectors.

5.3. Genetic immunopotentiation requires to break immune tolerance to tumors

Modification of gene vectors to obtaining amplification and targeting is a critical goal of the strategies of mutation compensation and molecular chemotherapy to be successful. In contrast, for genetic immunopotentiation strategies, it may well be that a sophisticated vector is not needed to facilitate the otherwise inefficient transfer of DNA into cells.

5.3.1. Polynucleotide immunization

The possibility exist for eliciting potent, prolonged, and specific immune responses through the intramuscular injection of fragments of nucleic acid encoding tumor-associated antigens (224). This so called 'polynucleotide immunization' (PNI) approach offers several advantages with respect to classic protein immunization. First, synthesis of the antigen in eukaryotic cells *in vivo* is more likely to result in a protein that is correctly folded and with its antigenic domains adequately presented. Second, PNI elicits a $CD8^+$ cytotoxic T lymphocyte response in addition to a humoral response. Third, long term expression of the encoded antigen may favor long-lived immunity. Finally, safety concerns related to virus-derived vaccines are obviated. Polynucleotides in the form of both DNA and RNA can be used. For example, plasmid DNA encoding carcinoembryonic antigen, a non-transforming tumor-associated antigen, is being tested in a clinical protocol for colorectal cancer patients. Transforming tumor-associated antigens, such as erbB-2, may be encoded with RNA constructs that avoid the risk of integration of a potentially oncogenic sequence and are expressed only transiently. Once the antigen is expressed in myofibers, its presentation to the effector cells follows an unknown pathway, but is known to induce antibody production, T-cell proliferation, lymphokine release, generation of CTL, and delayed hypersensitivity reactions. Importantly, encouraging results in animal models have been followed by clinical trials for both immune protection and therapeutic applications. Although tumors are antigenically heterogeneous, the hypothesis is that immune responses against the polynucleotide-encoded antigens can break immune tolerance for the tumor via a single epitope, which, in turn, would alert the immune system to the existence of the tumor as a foreign entity, provoking a systemic response.

5.3.2. Danger versus tolerance

The classical paradigm of tumor immunology considers the responses of the immune system to follow a model of discrimination between 'self' and 'non-self' antigens. However, an alternative model has been proposed, according to which the key fact for initiating an efficient immune response is the detection by the host of 'danger' (for instance, the beginning of either an inflammatory reaction or tissue damage) (201). This new model considers the location and kinetics of antigen presentation to the immune system as an alternative signal that can modulate the immune response (215). Thus, the presentation of the (tumor) antigen in the lymph node environment by antigen presenting cells activated in the tumor would be critical for an efficient response (174). This

model can change the emphasis applied in immunotherapy strategies. Whereas in the classical model importance is given to the identification of tumor antigens and elaboration of vaccines based on these antigens, new goals potentially more relevant could be to orchestrate inflammatory processes in tumor foci, to activate dendritic cells and other antigen presenting cells, and to drive the migration of T lymphocytes towards the tumor. In other words, the aim should be to recruit not only the adaptive immune response but also and most importantly the cells (macrophages, neutrophils, NK cells) and mediators (cytokines, chemokines) of the innate immune system (201).

5.3.3. Enhanced antigen presentation

As we reviewed, many tumors are ignored by the immune system. Thus, tumor antigen-specific T lymphocytes, which are present in the immune repertoire, are not activated and migrate systemically without showing any special tropism towards its cognate antigens in the tumor. This has been attributed to a lack of functional dendritic cells (DCs) in tumors (225). Indeed, DCs infiltrating several tumors lack B7-1 and B7-2 molecules, which reveals a non-stimulatory status and impedes the encounter by T lymphocytes of the required signal 2 for antigen-specific activation. However, when DCs are exposed *ex vivo* to tumor antigens and these DCs are then reinfused, CTL activation ensues. In animal models, this intervention achieves a protective effect against subsequent exposure to tumors and also can induce a therapeutic effect in tumors already present (226). This strategy is currently being explored in patients (227; 228). Multiple vectors are being tested for delivering tumor antigens into DCs, including viral vectors, naked DNA, RNA, tumor lysates, and peptides. It is probable that methods that maximize exposure of DCs to a variety of tumor antigens may have an advantage by overcoming the expected emergence of antigen-loss variants as well as natural immunovariation of tumors. Such a principle has been powerfully accomplished in animal studies by generating fusions of DCs and tumor cells (229). This strategy has been tested in transgenic animals tolerant to the antigen MUC1, and refractory to vaccination with irradiated MUC-1 positive cells. Immunization with the dendritic cell fusions that express MUC1 resulted in the rejection of established metastases and no apparent autoimmunity against normal tissues. These findings demonstrate that tolerance to tumor-associated antigens can be reversed, and suggest that immunization with hybrids of dendritic and carcinoma cells may be a powerful methodology for whole cell vaccination against cancer.

Conclusion

The delineation of the molecular basis of cancer allows for the possibility of specific intervention at the molecular level for therapeutic purposes. To this end, three main approaches have been developed: mutation compensation, molecular chemotherapy, and genetic immunopotentiation. For each of these conceptual approaches, human clinical protocols have entered testing in Phase I and II to assess dose escalation, safety, and toxicity issues, and more recently to evaluate efficacy, respectively. However, major problems remain to be solved before these approaches can become effective and common place strategies for cancer. Principle among these is the basic ability to deliver therapeutic genes quantitatively, and specifically, not only into tumor cells but also into tumor supporting tissues and effector cells of the immune system. As vector technology fulfills these stringent requirements, it is anticipated that the promising results already observed in pre-clinical studies will translate quickly into the clinic for amelioration of life-threatening tumor diseases.

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Obstacles to Curing Cancer Imposed by Tumor Cells	Potential Contribution of Gene Transfer
1. Tumors are genetically unstable, and thus they are extraordinarily adaptable to environmental changes (This is arguably the biggest obstacle, and has never been approached directly.)	Gene transfer of DNA repair or cell cycle checkpoint genes that restore DNA stability
2. Tumors are heterogeneous in many respects, including genetic mutations, expression of oncoproteins, immunogenicity, response to environmental changes, etc.	Targeting of "genetically homogeneous" tissues, such as the tumor vasculature; genetic immunopotentiation
3. As a consequence of Obstacles #1 and #2, tumors have, or acquire, resistance to cellular toxins and to many other therapeutically induced cellular insults	Strategies above, associated with chemotherapy or radiotherapy or with the transfer of genes that sensitize tumor cells to drugs or radiation
4. Tumors can have a low cellular growth fraction; therefore they are less susceptible to mitotic toxins and to gene transfer vectors that require dividing cells	Use of vectors that do not require cellular division for gene delivery and expression (adenovirus, herpesvirus, lentivirus, chimeric systems); repeated administration of non-immunogenic vectors
5. Tumors form metastases, which have to be reached systemically to eradicate the tumor	Use of targetable, injectable vectors (tropism-modified viruses, cellular vehicles); genetic immunopotentiation
6. Tumors do not express specific tumor antigens or immune costimulatory molecules; alternatively, tumors down-regulate immune recognition, induce tolerance, and inhibit the immune response	Transfer of genes encoding costimulatory molecules and cytokines; genetic modification of antigen-presenting cells; induce inflammatory reactions that activate antigen presentation; transfer of genes blocking tumor-secreted inhibitors of the immune response
7. The spontaneous behavior of human tumors is somewhat different from that of malignant cells in vitro, and from that of experimental tumors in animal models	Development of better animal models, including tumor models in transgenic mice
8. Tumors are diagnosed in advanced stages, when billions of tumor cells exist in the body, frequently widely disseminated	Development of amplification vector systems (replicative viral vectors); use of targetable, injectable vectors; genetic immunopotentiation
9. The understanding and treatment of cancer requires the contribution of very diverse fields of basic knowledge, biotechnology, and medical practice	De facto multidisciplinary recruitment of gene therapy researchers

Table 1. Potential Contributions of Gene Therapy against the Obstacles for Curing Cancer

Strategy	Clinical Trials*	Molecular Mechanism of Anticancer Effect
Mutation compensation	6	Inhibition of expression of dominant oncogenes
	14	Augmentation of deficient tumor-suppressor genes
	2	Abrogation of autocrine growth factor loops (single chain antibodies)
Molecular chemotherapy	26	Selective delivery of toxin or toxin gene to cancer cells
	8	Chemoprotection of normal tissues during high-dose chemotherapy
Genetic immunopotentiation	49	In vitro transduction—augmentation of tropism or cell killing capacity of tumor-infiltrating lymphocytes; genetic modification of irradiated tumor cells
	40	In vivo transduction—administration of costimulatory molecules or cytokines; immunization with virus encoding tumor-associated antigens
Viral-mediated oncolysis	2	Tumor cell lysis by viral vector replication

* Registered in the NIH Office of Recombinant DNA Activities in the first half of 1998 (http://www.nih.gov/od/orda/protocol.htm)

Table 2. Clinical Trials of Gene Therapy for the Treatment of Cancer

Type	Vector System	Duration of Expression	Clinical Trials (#)	Distinguishing Features
Nonviral	Liposomes	Transient	30	Repetitive and safe administration feasible, inefficient gene delivery, transient expression
	Naked DNA or RNA (injection, gene gun, electroporation)	Transient	5	Easy preparation, inefficient gene delivery, transient expression
	Molecular conjugates	Transient	-	Flexible design, inefficient gene delivery, transient expression, unstable in vivo
Viral	Retrovirus	Prolonged	63	Integrates into the chromosome of dividing cells, unstable in vivo
	Adenovirus	Transient	33	Highly efficient in vivo, production in high titer, tropism can be modified, induces potent inflammation and immunity, replicative vectors available
	Poxvirus (vaccinia)	Transient	15	Extensive clinical experience with parent virus, large insert capacity, induces potent inflammation and immunity
	Adeno-associated virus	Prolonged	-	Non pathogenic, low insert capacity, difficult to scale-up
	Herpes simplex virus	Transient	1	Highly efficient in vivo, large insert capacity, cytotoxic, replicative vectors available
	Chimeric vectors (e.g., Ad/Retro)	Prolonged	-	Combine features of component genetic vectors
	Lentivirus	Prolonged	-	Integrates into the chromosome of *both* dividing and nondividing cells, well-characterized production system not yet established

* Registered in the NIH Office of Recombinant DNA Activities in the first half of 1998 (http://www.nih.gov/od/orda/protocol.htm)

Table 3. Gene Transfer Systems used Clinically against Cancer

Target*	Strategy	Vector	Tumor type
p53	Replacement of tumor suppressor gene	Adenovirus	Non-small cell lung cancer, head and neck squamous cell carcinoma, hepatic metastases of colon cancer, hepatocellular carcinoma, prostate cancer, breast cancer
RB (Retinoblastoma)	Replacement of tumor suppressor gene	Adenovirus	Bladder cancer
BRCA-1	Replacement of tumor suppressor gene	Retrovirus	Ovarian cancer
erBb-2	Inhibition of promoter by E1A	Cationic liposome complex	Breast and ovarian cancers overexpressing erbB-2
Insulin-like growth factor 1	Blockade by antisense	Cationic liposome complex	Glioblastomas
k-ras	Blockade by antisense	Retrovirus	Non-small cell lung cancer
c-myc	Blockade by antisense	Retrovirus	Breast and prostate cancers
TGFβ	Blockade by antisense	Plasmid & electroporation	Glioblastoma
erBb-2	scFv	Adenovirus	Ovarian cancer

* Registered in the NIH Office of Recombinant DNA Activities in the first half of 1998 (http://www.nih.gov/od/orda/protocol.htm)
scFv: single-chain intracellular antibody

Table 4. Mutations Compensation Strategies Used Clinically

Enzyme (origin)	Prodrug	*In vitro* bystander effect	Target cell	Distinguising features	Refs.
Carboxylesterase (rabbit liver)	CPT-11 (irinotecan)	+++	Dividing cells	Prodrug already in clinical use	91, 92
Carboxypeptidase G2 (bacterial)	CMDA	+++	Dividing and non-dividing cells	Does not require cell cycling	93
Cytochrome P-450 isoenzyme (rat liver)	Cyclophosphamide, isophosphamide	Yes	Dividing cells	Brain intratumor administration allows generation of toxic metabolites that usually do not cross blood-brain barrier	94
Cytosine deaminase (*E. coli*)	5-fluorocytosine	++ (independent of cell contact)	Dividing and possibly non-dividing cells	Intense sensitization	95-98
Deoxycytidine kinase (human)	cytosine arabinoside	+ (dependent on cell contact)	Dividing cells	Human origin of gene avoids an immune response	99, 100
Nitroreductase (*E. coli*)	CB1954	+++ (independent of cell contact)	Dividing and non-dividing cells	Does not require cell cycling	101-104
Purine nucleoside phosphorylase (*DeoD* gene of *E. coli*)	MeP-dR	+++	Dividing and non-dividing cells	Most intense bystander effect	105
Xantine-guaninephosphoribosyl transferase (*gpt* gene of *E. coli*)	6-thioxanthine, 6-thioguanine	++ (independent of cell contact)	Dividing cells	*gpt* is also a drug resistance gene	106-108
Thymidine kinase (herpes simplex virus)	ganciclovir		Dividing cells	Most extensively used in clinical trials	69
Thymidine kinase (varicella zoster virus)	BVDU	++ (dependent on cell contact)	Dividing cells	-	109

* BVDU: (E)-5-(2-bromovinyl)-2'-deoxyuridine; CB1954: 5-(aziridin-1-yl)-2,4-dinitrobenzamide; CMDA: 4-[(2-chloroetyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid; Mep-dR: 9-(beta-D-2-deoxyerythropentofuranosyl)-6-methylpurine. Transduced cells needed for complete cell growth inhibition in cell mixing experiments: 10% or less: +++; from 10 to 50%: ++; more than 50%: +

Table 5. Prodrug/enzyme Combinations for Molecular Chemotherapy

Principle	Basis	Strategies	Examples of applications
Selective delivery (transductional targeting)	Anatomically directed administration	Intratumoral, intravascular, or body compartment injection of vector	Injection of a plasmid encoding the immunostimulatory molecule B7 in foci of malignant melanoma (114); intraarterial infusion of a plasmid encoding VEGF in an ischemic vascular tree (115); intraperitoneal administration of adenovirus expressing the suicide gene *tk* in model of ovarian cancer (116)
	Target cell physiology	Exploit cell cycle differences: gene transfer by retroviruses occurs only in dividing cells	Brain tumors (tumors are surrounded by neural cells, which are non mitotic) (117)
		Exploit cell cycle differences: Herpes virus deleted in *tk* or other genes replicates only in cells undergoing division	Injection of virus is followed by lytic replication in liver metastasis (118) and brain tumors (119) (tumors are surrounded by cells that are non mitotic)
		Exploit differences between transformed and normal cells: Adenovirus deleted in E1B gene replicates selectively in p53 defective tumor cells	Injection of virus is followed by lytic replication in head and neck tumor cells (120)
	Specific receptors in target cells	Retrovirus: pseudotyped retroviruses (built with heterologous envelope proteins that confer novel tropism); retroviruses with genetically, chemically, or immunologically modified envelope proteins	Retrovirus with the genome and core of Murine Leukemia Virus and the envelope protein of Vesicular Stomatitis Virus have wider tropism (121)
			Retrovirus with modified envelope protein that includes a fragment of erythropoyetin (122) or a single-chain variable region directed against known epitopes such as MHC class I molecules (123) or CEA (124)
		Adenovirus: genetic modifications in the fiber; immunologically-mediated attachment of cellular ligands	The modification of the adenovirus fiber is feasible ((125, 126). Adenovirus with folate (126) or the fibroblast growth factor (FGF) attached to the virus via anti-knob Fab antibody fragments in ovarian tumors (128)
		Molecular conjugates that combine a DNA binding domain and a cellular ligand	Asialoglycoprotein-based conjugates target hepatocytes (129); adenovirus enhances transduction efficiency of conjugates (130) transferrin-based conjugates target leukemic cells (131)
		Liposomes modified with antibodies specific against cellular receptors	Liposomes coupled with antibodiy G22-MCA against glioma cells have higher transduction efficiency (132)
Selective expression (transcriptional targeting)	Tumor specific promoters	A suicide gene is administered under the control of a promoter sequence that is active on tumor cells	Adenoviruses expressing *tk* under the alpha-fetoprotein promoter in hepatoma (133), or DF3 (MUC1) in breast cancer (134). Also shown with cells stably expressing the CD gene under the CEA promoter in colon cancer (135), and with cells transfected with the SLPI promoter, which is expressed in several carcinomas, to direct transcription of *tk* (136). Same principle shown with promoter of erbB-2, overexpressed in breast and pancreatic tumors (137)
	Tissue specific promoters	A suicide gene is administered under the control of a promoter sequence that is active in a particular tissue	Tyrosinase (melanocytes) directs expression of *tk* into malignant melanoma (138) and other melanocytes; surfactant protein-A drives *tk* expression in lung cancer cell lines (139)

CD: cytosine deaminase; SLPI: secretory leukoprotease inhibitor; *tk*: thymidine kinase; VEGF: vascular endothelial growth factor

Table 6. Targeting of Cancer Gene Therapy